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INFLUENCE OF VARIATIONS OF MEDIA ON ACID PRODUCTION BY STREPTOCOCCI.

By F. S. JONES, V.M.D.

(From the Department of Animal Pathology of The Rockefeller Institute for Medical Research, Princeton, N. J.)

(Received for publication, May 5, 1920.)

In an attempt to differentiate species of organisms closely resembling each other, the fermentation of carbohydrates and other substances plays an important part. When the fermentation characters of streptococci were first studied indicators such as litmus were employed which revealed only qualitative changes. Later quantitative determinations of acid by titration against solutions of alkali came into use.

More recently Clark and Lubs[1] have employed the hydrogen ion concentration method as an aid in the differentiation of closely allied species of organisms. Several have applied it for determination of acid production by streptococci. The advocates of the newer method point out that it is more accurate than titration since it indicates only free acids. The term "limiting or final hydrogen ion concentration" has come into use, since it indicates the maximum acidity or alkalinity produced by a given organism. Thus Avery and Cullen[2] have shown that human streptococci grown in veal infusion bouillon containing 1 per cent dextrose attained a limiting hydrogen ion concentration of 5.0 to 5.2. The bovine group produced more acid, 4.3 to 4.5. Ayers, Johnson, and Davis[3] some time previously had grown human hemolytic streptococci in a desiccated yeast-peptone-dextrose medium, one part of each per 100 parts of water, and found that the bulk of their strains reached a final hydrogen ion concentration of 5.4 to 6.0; 5.6 seems to have been the average. Smillie,[4] who used 1 per cent dextrose in fermented veal infusion bouillon, records the figures pH 5.1 to 5.4 for a few human strains. Brown[5] observed a limiting acidity of pH 5.1 to 5.4 for human streptococci grown in plain bouillon containing 1 per cent dextrose.

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[1] Clark, W. M., and Lubs, H. A., *J. Bacteriol.*, 1917, ii, 1.

[2] Avery, O. T., and Cullen, G. E., *J. Exp. Med.*, 1919, xxix, 215.

[3] Ayers, S. H., Johnson, W. T., and Davis, B. J., *J. Infect. Dis.*, 1918, xxiii, 290.

[4] Smillie, W. G., *J. Infect. Dis.*, 1917, xx, 45.

[5] Brown, J. H., *J. Exp. Med.*, 1920, xxxi, 35.

Fennel and Fisher[6] record the acid limit of *Streptococcus hæmolyticus* as pH 4.5. Whether these figures include both the human and bovine varieties is not stated.

Since considerable variation in acid production had been observed by various workers it seemed possible that the differences might be the result of variations in the media. Broadhurst[7] brought out the difference in the amount of titratable acid produced by streptococci grown in broth prepared from meat and meat extract. Streptococci grown in the former media produced two or three times as much acid as those grown in meat extract media. Many have observed the marked increase in the amount of titratable acid when more than 1 per cent of peptone was used in sugar broths. The writer has frequently noted that streptococci grown in fermented bouillon containing 1 per cent dextrose to which sterile serum had been added invariably produced more titratable acid than cultures in the same media which did not contain serum. A small number of tests also showed that the hydrogen ion concentration was often greater in the dextrose serum broth. The results, then, were not readily explicable on the assumption that the buffer activity of the serum was responsible for the increase in the acidity. It seemed desirable to note the effect of variations of the medium upon acid production.

Veal infusion was prepared from the flesh of a calf 1 day old. It is customary in this laboratory to add two parts of water to one part of the ground flesh. The infusion was divided. To one portion 1 per cent of peptone (Fairchild's) and 0.5 per cent of sodium chloride were added and the reaction was adjusted to + 0.8 (pH 7.6). The remainder of the infusion was strained and inoculated with a young culture of *Bacillus coli* and permitted to ferment at 38° C. for 18 hours. After straining through cotton and gauze, the usual quantity of peptone and salt was added to the larger portion of the filtrate. The reaction was adjusted to + 0.8 (pH 7.6). To a smaller portion of the fermented infusion 2 per cent of peptone and the usual amount of sodium chloride were added. This then provided three types of bouillon prepared from the same materials, unfermented broth and fermented bouillon containing 1 and 2 per cent of peptone respec-

[6] Fennel, E. A., and Fisher, M. B., *J. Infect. Dis.*, 1919, xxv, 444.
[7] Broadhurst, J., *J. Infect. Dis.*, 1913, xiii, 404.

tively. The broths were tubed in amounts of 13 cc. After sterilization under pressure, 1 cc. of a sterile 13 per cent solution of dextrose was added to each. The columns of liquid in the tubes ranged from 6 to 6.5 cm. in height. To half of the tubes of fermented and half of the tubes of unfermented bouillon, 0.5 cc. of sterile horse serum per tube was added. The 2 per cent peptone-dextrose fermented broth was used without other additions.

All cultures employed in the experiment produced hemolysis (beta) in agar plates containing 8 per cent of defibrinated horse blood. All the human streptococci[8] had been grown on artificial media for 2 or more years. All had been isolated from diseased conditions. The bovine strains were isolated by the writer. Five were from cases of mastitis. Two were found in market milk, but were identical in all respects with mastitis streptococci. The equine streptococci, with one exception, were isolated from the nasal mucosa and pharynx of horses suffering from influenza. Equine Streptococcus H.A. 2 was cultivated from an abscess. The low acid-producing streptococci were isolated from market milk. Recent investigations[9] have shown that these may be carried in small numbers in apparently normal udders. Strains M.J. 1 and M. 53 were isolated in 1917. The others were first cultivated in the summer of 1918.

Each tube was inoculated with 0.1 cc. of an 18 hour broth culture. All were incubated at 38°C. for 10 days, when hydrogen ion determinations[10] and titrations against 0.05 N sodium hydroxide were made. Maximum growth was always obtained in the media containing serum. The plain and fermented bouillon cultures were about as vigorous as those observed in the 2 per cent peptone medium. The low acid-producing streptococci from milk grew poorly in media without serum. Serum frequently changes the character of the growth. Strains which grow only at the bottom of tubes in the plain broth frequently produce a marked turbidity throughout the serum medium.

[8] The writer is indebted to Dr. J. Howard Brown, of this Department, for Cultures 32, S.H., S. 8, and 40. Dr. O. T. Avery, of the Hospital of The Rockefeller Institute, supplied Cultures 1, 20, and 24.

[9] Jones, F. S., *J. Exp. Med.*, 1920, xxxi, 347.

[10] Dr. P. E. Howe, of this Department, prepared the standards and indicators for this experiment.

In Tables I to IV the acid production by the different groups of streptococci is given. The figures under the titration columns represent the actual acidity reached in the tubes.

TABLE I.

*Acid Production by Human Streptococci in Plain and Fermented Broths.**

Strain No.	Fermented bouillon.		Fermented bouillon and serum.		Fermented bouillon containing 2 per cent peptone.		Plain bouillon.		Plain bouillon and serum.	
	pH	Titration.	pH	Titration.	pH	Titration.	pH	Titration.	pH	Titration.
		per cent		*per cent*		*per cent*		*per cent*		*per cent*
32	5.5	3.6	5.2	4.2	5.7	4.5	5.1	4.2	5.0	4.8
S.H.	5.5	4.2	5.2	4.5	5.5	5.0	5.2	4.0	4.9	4.6
S. 8	5.8	3.9	5.2	4.1	6.0	3.9	5.6	3.2	4.9	4.2
40	5.2	4.2	5.0	4.6	5.4	4.9	5.1	4.1	5.0	4.7
1	5.8	3.5	5.2	4.2	5.8	3.9	5.5	3.3	5.0	4.4
20	5.7	3.1	5.2	4.0	5.7	3.9	5.2	3.2	5.1	4.4
24	5.7	3.1	5.1	4.2	5.7	4.3	5.1	3.7	4.9	4.5

* All the media contained 1 per cent dextrose.

TABLE II.

Acid Production by Bovine Streptococci in Plain and Fermented Broths.

Strain No.	Fermented bouillon.		Fermented bouillon and serum.		Fermented bouillon containing 2 per cent peptone.		Plain bouillon.		Plain bouillon and serum.	
	pH	Titration.	pH	Titration.	pH	Titration.	pH	Titration.	pH	Titration.
		per cent		*per cent*		*per cent*		*per cent*		*per cent*
B.M. 1	4.6	6.1	4.6	6.2	4.6	7.0	4.6	5.7	4.6	5.9
C. 59	4.8	5.7	4.8	6.2	5.0	7.0	4.6	6.2	4.6	6.1
B.M. 24	4.9	5.4	4.8	6.1	4.9	6.7	4.6	5.8	4.5	5.7
M. 26	4.8	5.5	4.7	6.3	4.8	7.1	4.6	5.8	4.5	5.7
" 43	4.7	6.0	4.6	6.5	4.7	7.2	4.6	5.9	4.6	5.9
C. 53	4.8	5.3	4.7	6.0	4.8	6.8	4.6	5.5	4.5	5.9
" 67C	4.8	6.2	4.8	6.5	4.8	7.4	4.6	6.0	4.6	5.9

Curves (Text-figs. 1 and 2) constructed from averages of Tables I to IV reveal considerable differences in acid formation in the various media. This is particularly true in the instance of the human and low acid-producing streptococci from milk. It will be observed that

these organisms grown in media low in nutritive material (fermented and plain broth), even though the media contain sufficient carbohydrate, fail to produce as much acid as when a richer medium is employed. The addition of serum, then, not only increases the titratable acid but the ionized acid as well. Thus in the richest medium

TABLE III.

Acid Production by Equine Streptococci in Plain and Fermented Broths.

Strain No.	Fermented bouillon.		Fermented bouillon and serum.		Fermented bouillon containing 2 per cent peptone.		Plain bouillon.		Plain bouillon and serum.	
	pH	Titration.	pH	Titration.	pH	Titration.	pH	Titration.	pH	Titration.
		per cent		*per cent*		*per cent*		*per cent*		*per cent*
In. 22	5.1	4.4	5.5	4.4	5.0	5.5	4.9	4.4	5.1	4.6
H.A. 2	5.1	4.3	5.5	3.2	5.1	5.3	4.7	4.4	5.0	4.6
In. 49	5.1	4.5	5.3	5.0	5.1	5.7	4.9	4.4	5.1	4.8
" 14	5.1	3.9	5.4	4.4	5.0	4.8	4.9	3.9	5.1	4.6
" 2	5.1	4.7	5.4	4.2	5.1	5.6	4.9	4.6	5.1	4.5

TABLE IV.

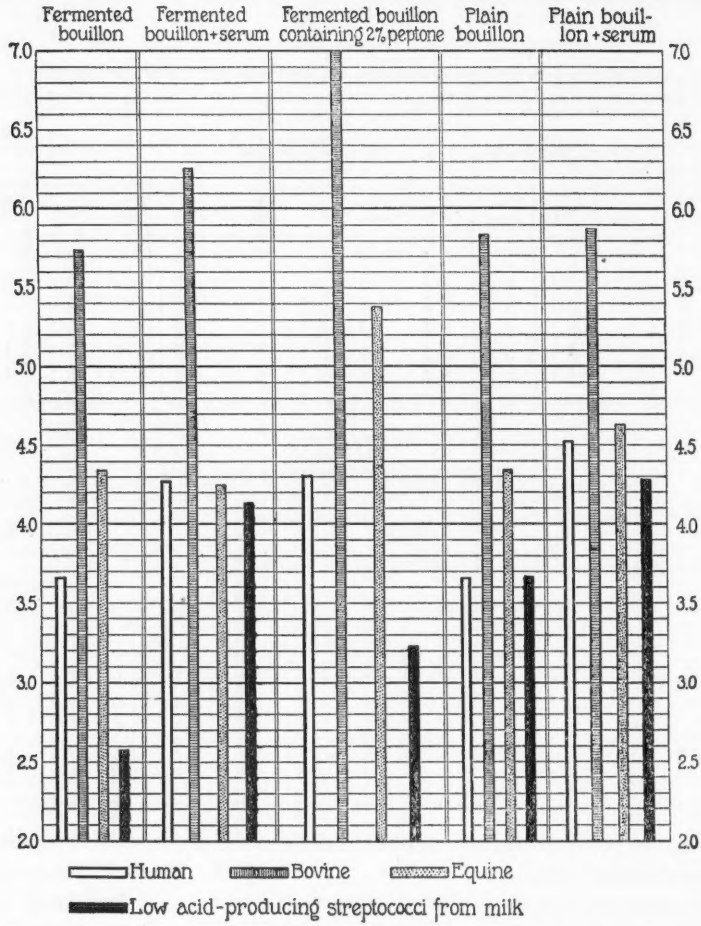
Low Acid-Producing Streptococci from Milk.

Strain No.	Fermented bouillon.		Fermented bouillon and serum.		Fermented bouillon containing 2 per cent peptone.		Plain bouillon.		Plain bouillon and serum.	
	pH	Titration.	pH	Titration.	pH	Titration.	pH	Titration.	pH	Titration.
		per cent		*per cent*		*per cent*		*per cent*		*per cent*
M.J. 1	6.3	2.6	5.4	4.1	6.5	3.1	5.2	4.0	5.2	4.0
M. 53	6.3	2.7	5.8	3.5	6.3	2.4	5.8	3.0	5.2	4.1
B.M. 30	6.6	2.0	5.2	4.1	5.9	3.7	5.1	4.0	5.1	4.3
" 22	5.9	3.1	5.1	4.6	6.3	3.7	5.5	3.4	5.1	4.6
" 60	6.3	2.4	5.1	4.4	6.3	3.2	5.5	3.9	5.1	4.4

(dextrose bouillon and serum) the maximum acid production is reached. The sharpest differences are brought out by the low acid-producing milk streptococci; here the greatest increases in acid formation are seen in the serum media. The fermented broth containing 2 per cent peptone apparently offers for the human and low acid-producing streptococci no more favorable media for acid production than that

containing 1 per cent peptone. One would expect the titratable acid to increase on account of the great buffer activity of peptone.

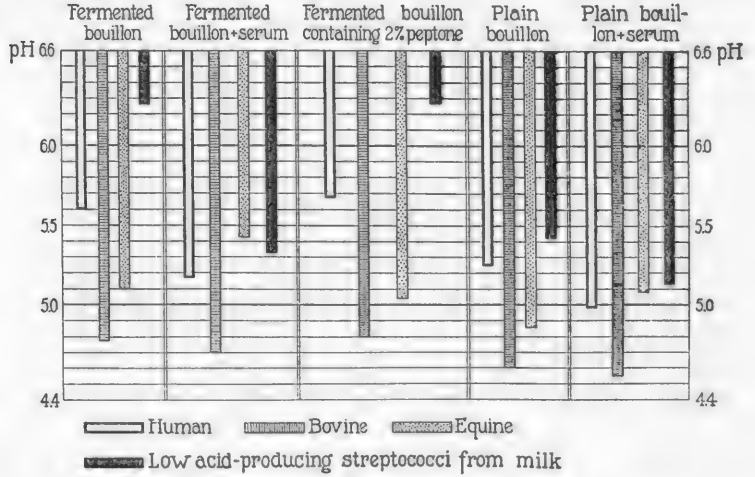
The bovine streptococci follow a somewhat similar curve. Apparently organisms of this type are able to utilize carbohydrate to



TEXT-FIG. 1. Titratable acidity produced by human, bovine, and equine streptococci and low acid-producing streptococci from milk when grown in various media.

a greater degree in media of lower nutritive value. The titratable acidity varies over a considerable latitude; the buffer effect of the 2 per cent peptone is marked. On the whole, there is more acid produced in the serum media.

The reverse is true of the equine streptococci. The organisms apparently produce more acid in media which do not contain serum. Broth containing 2 per cent peptone affords a medium equally as good for acid production as either of the other bouillons to which serum was not added. Two possible explanations for the failure of the



TEXT-FIG. 2. Limiting or final hydrogen ion concentration of human, bovine, and equine streptococci and low acid-producing streptococci from milk when grown in various media.

serum to increase the production of acid suggest themselves. The equine streptococci may not require serum; peptone and meat extracts may be sufficient for all purposes. On the other hand, normal horse serum may contain some substance antagonistic to growth. It is admitted that all strains grew well in the serum media.

The experiment was repeated with the same cultures, but with broths made from the flesh of different calves. When bouillon prepared from the flesh of a calf 6 weeks old was employed much the same results were obtained.

DISCUSSION.

The results obtained readily show that the limiting hydrogen ion concentration may be influenced by differences in the media. The question naturally arises as to the final or limiting hydrogen ion concentration reached by an organism. From present knowledge it may be defined as the acid production in a given medium which finally limits the growth of the organism. That such figures vary over a considerable latitude must be admitted, since human streptococci in one medium may produce acid to the value of pH 5.6 and in another to pH 4.9. Avery and Cullen's figures represent the maximum acid production for human (pH 5.0 to 5.2) and bovine (pH 4.3 to 4.5) streptococci in a medium most favorable for growth. Ayers, Johnson, and Davis' figures (5.5 to 6.0), on the other hand, are given as the final hydrogen ion concentration in a medium of low nutritive value for pathogenic streptococci. While these data were being gathered together the paper of H. Jones[11] appeared. He obtained even greater variations than those recorded in the preceding protocols. He showed that a culture of *Streptococcus hæmolyticus* when grown in glucose broth produced acid to the value of pH 5.11, but when ascitic fluid was added to the bouillon an acidity of pH 4.63 was reached. The increase in acidity failed to vary with an increase in the amount of enriching material. The same limiting hydrogen ion concentration was reached in media to which varying amounts (3 drops to 3 cc.) of ascitic fluid had been added. H. Jones also observed that the initial reaction of the media may influence the limiting hydrogen ion concentration. Thus in concluding it is pointed out that the limiting hydrogen ion concentration of an organism should be defined in terms of media composition, the initial reaction, and other conditions which may favor or hinder abundant growth.

Clark and Lubs[12] noted that methyl red may be destroyed in a short time by active cultures of *Bacillus coli*. Others had observed the same for nitrifying bacteria. Certain streptococci, especially those from sour milk, exhibit this activity to a marked degree while in the active growth phase. The indicator begins to fade within 5 or 10 minutes and at the end of an hour is completely decolorized.

[11] Jones, Horry, *J. Infect. Dis.*, 1920, xxvi, 160.

[12] Clark, W. M., and Lubs, H. A., *J. Bacteriol.*, 1917, ii, 191.

The advocates of the exclusive application of the hydrogen ion concentration method to bacteriological study have frequently pointed out the unreliability of titration. In studying the tables and charts it will be observed that the differences in titratable acidity are clear-cut. Even the addition of 2 per cent peptone with its buffer effect still reveals great differences in the amount of acid formed by the different groups of organisms. From these experiments one is inclined to believe that titration is equally as satisfactory as the newer method for the study of the fermentative activity of streptococci.

The greatest differences in acid production are brought out in a relatively unfavorable medium, such as dextrose fermented broth containing 1 per cent peptone.

SUMMARY.

The results of variations in acid production in 1 per cent dextrose fermented and unfermented veal broth modified by the addition of 4 per cent of horse serum or 2 per cent of peptone have been recorded. Human streptococci and a group of low acid-producing streptococci from milk produce less acid in the simpler broths (fermented and unfermented). 2 per cent peptone fails to increase the amount of acid produced by these two groups.

The bovine streptococci act much the same as those of human origin. The equine streptococci apparently do not require serum in addition to carbohydrate since they tend to produce less acid in serum media.

The following figures indicate the average minimum and maximum acid production of the various streptococci under the conditions set forth in the experiment.

Human: pH 4.97 to 5.66; titratable acidity 4.51 to 3.66.

Bovine: pH 4.56 to 4.77; titratable acidity 7.0 to 5.74.

Equine: pH 4.86 to 5.42; titratable acidity 5.38 to 4.24.

Low acid-producing streptococci from milk: pH 6.28 to 5.14; titratable acidity 2.56 to 4.28.

ENHANCEMENT OF THE OPSONIZING AND AGGLUTINATING POWERS OF ANTIPNEUMOCOCCUS SERUM BY SPECIFIC PRECIPITATING SERUM.

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(Received for publication, May 3, 1920.)

Bordet,[1] Ehrlich and Morganroth,[2] Pfeiffer and Friedberger,[3] and others have shown that it is possible to produce in animals, through the injection of certain immune or normal sera, antisera capable of inhibiting the action of antibodies. The action of such sera has been ascribed to specific substances designated as anti-antibodies, which are formed when animals of one species are injected with the serum of another species. The serum of the injected animals inhibits the action of antibodies derived from the species whose serum was used as antigen, but is ineffective against antibodies derived from another species. A number of anti-antibodies have been thus produced; *viz.*, antihemolysins, antiprecipitins, antihemagglutinins, anticomplement, and antibacteriolysins. Attempts to produce anti-antitoxins and antibodies for bacterial agglutinins have not been successful. Ehrlich and Morganroth[4] and Wassermann[5] maintain that anti-antibodies for bacterial antibodies cannot be formed in the animal body since animal tissues do not possess receptors suitable for bacterial antibodies. Pfeiffer and Friedberger,[3] however, report experiments in which they succeeded in obtaining a very effective inhibiting serum for cholera vibriolysins.

On the assumption that bacterial anti-antibodies may theoretically be formed in the animal body, the question becomes of interest in connection with the use of therapeutic sera in man, particularly when the same individual is given repeated injections of serum from the same animal species. This condition frequently arises, since therapeutic sera are almost exclusively derived from the horse.

[1] Bordet, J., *Ann. Inst. Pasteur*, 1904, xviii, 591.

[2] Ehrlich, P., and Morganroth, J., *Berl. klin. Woch.*, 1901, xxxviii, 598.

[3] Pfeiffer, R., and Friedberger, E., *Berl. klin. Woch.*, 1902, xxxix, 4; *Centr. Bakteriol., 1te Abt., Orig.*, 1903, xxxiv, 70.

[4] Ehrlich, P., and Morganroth, J., *Berl. klin. Woch.*, 1901, xxxviii, 569.

[5] Wassermann, A., *Z. Hyg. u. Infectionskrankh.*, 1903, xlii, 267.

The observations here reported are the results obtained in experiments on the production of pneumococcus antiopsonins. Second infections due to the same type of pneumococcus, though rare, have occurred. If antiopsonins should arise following serum treatment of the first infection, and persist in the patient's blood, they would tend to neutralize the opsonins in the immune serum employed during the second attack, thus lessening its value. The same effect might theoretically be observed if the patient had at any previous time received normal or non-specific immune horse serum.[1]

As will be seen from the following experiments, no evidence of the formation of pneumococcus antiopsonins could be obtained. On the contrary, the opsonizing and agglutinating properties of the immune horse sera were greatly enhanced in the presence of the sera which were expected to contain inhibiting substances.

EXPERIMENTAL.

Monovalent pneumococcus horse sera, Types I, II, and III, obtained from the Hospital of The Rockefeller Institute, were injected into rabbits. Each rabbit received intravenously 8 cc. of serum of one of the three types, and after 14 days, when the injected serum had disappeared, as indicated by opsonic tests, the animals were bled and their sera collected. Dilutions in salt solution were made of the antipneumococcus horse sera, each equal to one-fourth the dilution finally desired, since equal volumes of whole rabbit serum, pneumococcus culture, and leucocytic suspension were to be added to it. In each of a series of serological tubes was placed 0.1 cc. of the undiluted serum of the injected rabbits, 0.1 cc. of antipneumococcus horse serum dilution, and 0.1 cc. of a 24 hour blood broth culture of the homologous pneumococci. After their contents had been thoroughly mixed by shaking, the tubes were placed in a water bath at 37°C. for 1 hour to allow for sensitization of the pneumococci and interaction of the rabbit and horse sera. At the end of this time there was added to each tube 0.1 cc. of a fresh suspension of leucocytes, obtained by injecting a guinea pig on the previous day intraperitoneally with aleuronat. The contents of the tubes were again thoroughly mixed and incubated for another hour in the water bath, when a film

was made from the sediment in each tube and the slides were fixed in methyl alcohol and stained with Manson's stain. Control experiments were run at the same time in which normal rabbit serum was substituted for that of the rabbits previously injected with pneumococcus horse serum.

To determine whether the order of combination would affect the result, three other methods of combining the various constituents were also employed as follows: (1) Pneumococcus horse serum and precipitating rabbit serum were incubated together for 1 hour before the addition of the pneumococci. After adding the pneumococci the mixture was incubated for another hour before adding the leucocytes. This method permitted considerable precipitation in the rabbit-horse serum mixture before sensitization of the bacteria was begun. (2) Pneumococcus horse serum and pneumococci were incubated for 1 hour before adding the precipitating rabbit serum. After adding the rabbit serum the mixture was incubated for 1 hour before adding the leucocytes. This method permitted sensitization of the bacteria before precipitation was begun. (3) Pneumococcus horse serum and homologous pneumococci were incubated together for 1 hour, when both the precipitating rabbit serum and the guinea pig leucocytes were added and the mixture was incubated for another hour.

No great difference in results was observed with these four methods, so that eventually only the first method, as described in detail above, was employed.

Experiment 1. Type I Pneumococcus Horse Serum.—Type I serum and pneumococci were combined, as described above, with the serum of rabbits previously injected with the Type I serum. The results obtained are presented in Table I.

From Table I it will be seen that the combinations described showed opsonization and microscopic agglutination in dilutions ten times as great as were effective when normal rabbit serum was substituted for that of the injected rabbits, while mixtures of the precipitating rabbit serum and normal horse serum gave uniformly negative results. When the pneumococcus horse serum was replaced by an equal volume of salt solution, neither normal nor precipitating rabbit serum was capable of causing the slightest opsonization or agglutination,

nor did these occur in control mixtures in which both rabbit and horse sera were replaced by salt solution. The Type I horse serum used in this experiment, when diluted with salt solution, showed microscopic agglutination and opsonization of Type I pneumococci in dilutions a little above 1:100.

TABLE I.

*Increased Opsonization and Agglutination of Type I Pneumococci When Combined with the Homologous Immune Serum in a Specific Precipitating Mixture.**

Tube No.	Type I antipneumococcus horse serum and normal horse serum dilutions.	Rabbit 1 (2 wks. after intravenous injection of 8 cc. of Type I antipneumococcus horse serum).				Normal rabbit serum.	
		With Type I antipneumococcus horse serum.		With normal horse serum.		With Type I antipneumococcus horse serum.	
		Agglutinins.	Opsonins.	Agglutinins.	Opsonins.	Agglutinins.	Opsonins.
1	1:100	++++	++++	−	−	++++	++++
2	1:300	++++	++++	−	−	++++	++++
3	1:500	++++	++++	−	−	−	−
4	1:1,000	++++	++++	−	−	−	−
5	1:2,000	++++	++++	−	−	−	−
6	1:3,000	++	++	−	−	−	−

* In this and succeeding tables the degree of opsonization and agglutination is represented by the number of plus signs, ++++ being complete, + slight but definite.

Experiment 2. Type II Pneumococcus Horse Serum.—The technique of Experiment 1 was employed, Type II serum and pneumococci being used. The results are given in Table II.

It will be seen from Table II that the results were essentially the same as those obtained in the experiments with Type I serum and pneumococci. In this particular experiment opsonization was marked in dilutions in which no microscopic agglutination was observed. This, however, was not always found, and was not peculiar to Type II, being sometimes encountered also in experiments with organisms and serum of Type I.

Type III Pneumococcus Horse Serum.—The opsonization and agglutination of Type III pneumococci by the homologous immune

horse serum was not augmented by the addition of the serum of rabbits injected with Type III horse serum, these organisms remaining unopsonized and unagglutinated except in very low dilutions of Type III serum, usually below 1:50.

TABLE II.

Increased Opsonization and Agglutination of Type II Pneumococci When Combined with the Homologous Immune Serum in a Specific Precipitating Mixture.

Tube No.	Type II antipneumococcus horse serum and normal horse serum dilutions.	Rabbit 2 (2 wks. after intravenous injection of 8 cc. of Type II antipneumococcus horse serum).				Normal rabbit serum.	
		With Type II antipneumococcus horse serum.		With normal horse serum.		With Type II antipneumococcus horse serum.	
		Agglutinins.	Opsonins.	Agglutinins.	Opsonins.	Agglutinins.	Opsonins.
1	1:100	++++	++++	−	−	++++	++++
2	1:300	+++	++++	−	−	+	+
3	1:500	++	+++	−	−	−	−
4	1:1,000	+	+++	−	−	−	−
5	1:2,000	−	++	−	−	−	−
6	1:3,000	−	+	−	−	−	−

Experiment 3. Normal Horse Serum.—All the experiments described above have in common the mixture of an immune pneumococcus horse serum with its homologous pneumococci in the presence of a specific precipitating serum. To determine whether the increase in opsonization and agglutination observed in these cases was due to some special property conferred upon the rabbit serum by the injection of the immune pneumococcus serum, or was merely dependent on the presence of the specific precipitating mixture, control experiments were run with the serum of rabbits injected with normal horse serum instead of the immune horse serum. One of these anti-horse rabbit sera had been prepared some months before, but still showed gross precipitins for horse serum in dilutions above 1 : 1,000. Two other anti-horse rabbit sera were prepared with two different samples of normal horse serum, and all three rabbit sera were tested with Type I and Type II pneumococcus horse serum and the homologous pneumococci. The results of these tests were similar for both Type I and Type II. A summary of the findings in an experiment with serum and organisms of Type I is given in Table III which will also serve to illustrate the results obtained with Type II serum and pneumococci.

It will be seen, from a comparison of Tables I and III, that the serum of Rabbit 3, injected some months previously with normal horse serum (Sample A) was but little better than normal rabbit serum in promoting the opsonization and agglutination of Type I pneumococci by the homologous serum, even though a specific precipitating mixture was here present. The other two rabbits, Nos. 4 and 5, injected with Samples B and C of normal horse serum, showed heightened opsonization and agglutination entirely comparable to that observed when Type I horse serum was combined with the serum

TABLE III.

Opsonization and Agglutination of Type I Pneumococci by the Homologous Immune Serum When Combined with the Sera of Rabbits Injected with Normal Horse Serum.

Tube No.	Type I pneumococcus horse serum dilutions.	Rabbit 3 (several mos. after intravenous injection of normal horse serum, Sample A).		Rabbit 4 (2 wks. after intravenous injection of normal horse serum, Sample B).		Rabbit 5 (2 wks. after intravenous injection of normal horse serum, Sample C).	
		Agglutinins.	Opsonins.	Agglutinins.	Opsonins.	Agglutinins.	Opsonins.
1	1:100	++	++	+++	++++	++	++++
2	1:500	+	+	++	++++	+	++++
3	1:1,000	−	−	+	++++	+	++++
4	1:2,000	−	−	+−	++	+	++

of rabbits previously injected with the same immune horse serum. Moreover, it was found that the sera of rabbits injected with Immune Horse Serum Type I, II, or III were equally effective in promoting the opsonization and agglutination of Type I and Type II pneumococci by the homologous horse sera, while organisms of Type III remained unaffected by any of the horse-rabbit serum combinations.

The failure of the serum of Rabbit 3 to produce the usual reaction is not understood. The serum of another rabbit immunized at the same time with the same sample of normal horse serum also failed to promote opsonization and agglutination of Type I and Type II pneumococci by the homologous immune sera.

It appears from the foregoing experiments that no special property is conferred upon the serum of rabbits by the injection of immune

horse serum, since no demonstrable antiopsonins are produced by such injections. On the contrary, the serum of such rabbits, as well as that of rabbits injected with normal horse serum, is capable, *in vitro*, of augmenting many fold the opsonizing and agglutinating power of Type I and Type II horse sera for the homologous pneumococci. These findings are in accord with the observations of Moreschi[6] on typhoid agglutinins. He found that when typhoid bacilli were subjected for 2 hours at 37°C. to the action of specific agglutinating serum from various sources (goat, man), and were then washed and resuspended in salt solution, the addition of a small quantity (0.05 cc.) of the serum of rabbits previously injected with the same agglutinating serum raised the titer of the immune serum five- to tenfold. His explanation for this phenomenon was that the serum of the injected rabbits, while itself incapable of agglutinating typhoid bacilli, combined with and precipitated the agglutinating serum already absorbed by the bacteria, thus causing agglutination. Non-specific bacteria, such as the cholera vibrio and *Bacillus dysenteriæ*, he found to be unaffected by the serum mixtures, while the precipitating serum had no affinity for unsensitized typhoid bacilli. The last two observations were also borne out by the present experiments, since in no case were Type I and Type II pneumococci opsonized or agglutinated by a heterologous or normal horse serum, even in the presence of a specific precipitating serum.

Protection Experiments.

Since the efficacy of therapeutic pneumococcus serum (Type I) depends largely on its opsonizing power, and since this power is so greatly enhanced *in vitro* by the addition of a specific precipitating serum, it was hoped that a corresponding increase in protective power might be produced by the same means. Experiments were accordingly carried out for the purpose of testing this point, mice being chosen as the test animals. The results obtained were disappointing. In this instance opsonization in the test-tube was not paralleled by protection in the animal, since no very marked increase in protection could be demonstrated. The impossibility of maintaining *in vivo*

[6] Moreschi, C., *Centr. Bakteriol., 1te Abt., Orig.*, 1908, xlvi, 456.

the same quantitative relations between bacteria and serum that exist *in vitro* may account for the failure. Dilution of the opsonizing and precipitating sera with peritoneal exudate and unequal absorption of the various constituents would tend to upset the balance very quickly, and no doubt would markedly affect the outcome. Incubation of the mixtures before injection served only to confuse the results, since the pneumococci were sometimes injured by the broth and died, while they multiplied in the tubes containing serum, so that not infrequently the control animals outlived the other mice. Only once was

TABLE IV.

*Protection Afforded to Mice from a Combination of Type I Pneumococcus Horse Serum with Specific Precipitating Rabbit Serum.**

Mouse No.	Weight.	Type I antipneumococcus horse serum.	Normal horse serum.	Normal rabbit serum.	Serum of rabbits previously injected with Type I horse serum.		Broth.	Type I pneumococcus broth culture.	Result.
					No. 1.	No. 6.			
	gm.	*cc.*	*cc.*	*cc.*	*cc.*	*cc.*	*cc.*	*cc.*	
1	20	0.0005		0.25				0.01	Died in 33 hrs.
2	20	0.0005			0.25			0.01	Survived.
3	20	0.0005				0.25		0.01	"
4	20	0.0005					0.25	0.01	Died in 22 hrs.
5	20		0.0005		0.25			0.01	" " 22 "
6	20		0.0005			0.25		0.01	" " 22 "

*The total volume of each dose was made up to 1 cc. and injected as soon as mixed into the peritoneal cavity, without incubation. The serum samples of Rabbits 1 and 6 were a month old at this time.

a successful experiment performed, in which the mice survived that had received a mixture of pneumococcus horse serum and specific precipitating rabbit serum, while all the other mice died. The protocol is presented in Table IV.

DISCUSSION.

From the foregoing experiments it appears that a simple precipitating mixture is capable of augmenting the opsonization and agglutination of Type I and Type II pneumococci by their homologous immune

sera. Similar observations have been made by other workers with other types of antibodies. Friedberger and Moreschi[7] found that rabbit red cells sensitized with goat amboceptor were much more readily hemolyzed in the presence of a small quantity of the serum of a rabbit previously injected with the goat amboceptor. Arkwright,[8] in his experiments on the agglutination of *Bacillus typhosus*, found that in a specific serum precipitating mixture, with bacterial extract as antigen, not only the specific bacteria, but even non-specific bacteria and inanimate particles were agglutinated. Nicolle[9] also reports that non-specific bacteria are carried down when present in a specific serum precipitating or agglutinating mixture. He found that in mixed suspensions containing equal numbers of *Bacillus coli* and *Bacillus typhosus*, the addition of an agglutinating serum specific for either would completely agglutinate both. The phenomena of non-specific opsonization and agglutination have not been observed in the present study, since neither Type I nor Type II pneumococci were opsonized or agglutinated in the presence of the heterologous immune serum or of normal horse serum, even though the addition of the anti-horse rabbit serum furnished a specific precipitating mixture. Arkwright's explanation for his results is that the coagulum formed in the serum precipitating mixture drew together and clumped the bacteria, specific and non-specific, a mechanical explanation that could hardly be applied to opsonization and phagocytosis. Since the reaction of an antigen with its specific antibody results in lowering the surface tension of the mixture,[10] and since it is thought that phagocytosis may be a surface tension phenomenon,[11] the explanation for the increased opsonization of Type I and Type II pneumococci by their homologous immune sera, in the presence of a specific precipitating serum, may perhaps lie in the changes in surface tension thus produced, though why a general lowering of the surface tension of the

[7] Friedberger, E., and Moreschi, C., *Centr. Bakteriol., Ite Abt., Orig.*, 1908, xlv, 346.

[8] Arkwright, J. A., *J. Hyg.*, 1914, xiv, 261.

[9] Nicolle, C., *Ann. Inst. Pasteur*, 1898, xii, 161.

[10] Wells, H. G., Chemical pathology, Philadelphia and London, 3rd edition, 1918, 208.

[11] Wells,[10] pp. 266–275.

medium should render the leucocytes more actively phagocytic is not clear. Another possibility is that precipitation takes place in the serum already absorbed by the sensitized bacteria, or that the previously precipitated substance is adsorbed by the bacteria together with the specific opsonins, and that these adsorbed substances render the pneumococci more susceptible to phagocytosis. It seems probable that the effect, whatever it may be, is exerted upon the bacteria, but at present no satisfactory explanation for the phenomenon has been reached.

SUMMARY.

1. No demonstrable antiopsonins are formed in rabbits following the intravenous injection of monovalent pneumococcus horse sera, Types I, II, and III.

2. The serum of rabbits injected with immune pneumococcus horse serum, Type I, II, or III, or with normal horse serum, when mixed in the proportion of 1:4 with Type I or Type II pneumococcus horse serum, can greatly augment, *in vitro*, the opsonization and agglutination of Type I and Type II pneumococci by the homologous immune horse sera. No similar effect is obtained with Type III serum and pneumococci.

3. The increase in opsonization and agglutination is dependent upon (*a*) specific sensitization of the pneumococci by the homologous immune serum and (*b*) the presence of the precipitating serum. In the absence of sensitization, as when a heterologous or normal horse serum is employed, opsonization and agglutination do not occur, even though a precipitating mixture is provided. The substitution of normal rabbit serum for the precipitating rabbit serum gives opsonization and agglutination in dilutions slightly higher than are effected with salt solution only, due possibly to the more favorable medium created for the leucocytes by the addition of 25 per cent of whole rabbit serum.

4. Different methods of combining the immune horse serum, precipitating rabbit serum, and pneumococci yield very similar results, preliminary sensitization of the bacteria before precipitation, or precipitation in the rabbit-horse serum mixture before the addition

of the pneumococci for sensitization causing little if any difference in result from that obtained when immune horse serum, precipitating rabbit serum, and pneumococci are all mixed and incubated together.

5. This increased opsonization in the test-tube does not seem to be paralleled by increased protective power, or at any rate such protection is not readily demonstrated.

USE OF THE SINGLE CELL METHOD IN OBTAINING PURE CULTURES OF ANAEROBES.

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Plate 23.

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The object of this work has been to determine to what extent the pipette method may be of service in the isolation of anaerobes. No question is raised as to the possibility of isolating anaerobes by plating or by any other of the methods ordinarily in use, but such methods are often time-consuming and sometimes leave the worker in doubt as to whether or not the culture is absolutely pure. Difficulties in isolation are especially great with mixtures of certain anaerobes, and doubt has been thrown on the results of some of the earlier workers because of the probability that their cultures were impure. The pipette method is relatively simple and rapid, and affords the worker the satisfaction of knowing that his culture is the progeny of a single cell. Further, it offers some advantages in the study of anaerobiosis, variability, motility, rate of growth, and behavior in a medium of small sowings exactly known.

Technique.

The technique followed in this work has been essentially that described for aerobes, except that the isolated organisms have been subsequently grown under anaerobic conditions. The pipette method has been fully described (1), and only such details will be given here as should be especially emphasized or are especially applicable to work with anaerobes.

The size of the isolation chamber may vary according to the needs of the investigator, but I have found most convenient one about 7 cm. long, 3.25 cm. broad, and 2 cm. high, which gives abundant working room and allows the use of a large cover-glass, 60 by 35 mm. The

preparation of the cover-glass is a matter of considerable importance, especially when colonies are to be grown on it. Carefully cleaned covers are smeared with vaseline and kept in stock ready for use. Just before use most of the vaseline is removed by holding the cover slantwise under the hot water tap. It is then washed with alcohol and again held under the tap. Finally, it is covered with alcohol and wiped clean with a cloth. It is then perfectly clear to the eye, but retains the very small film of vaseline so necessary to the preparation of small droplets. Prepared cover-glasses may be sterilized by dry heat or over the flame, care being taken to avoid heat sufficient to burn off the vaseline.

The point of the pipette should be made with an opening large enough to admit organisms readily, but not so large as to make it difficult to isolate the organisms in very small droplets. A diameter about equal to the average length of a spore-containing bacillus of *Bacillus tetani* is very satisfactory. It will be found most convenient to make very fine pointed closed pipettes and to break off portions of the point in a drop of broth under microscopic control until an opening of convenient size is obtained. A small quantity of broth should be allowed to enter the pipette before isolation is begun.

Certain doubts which have been raised as to the possibility of isolating a single organism in a hanging drop must arise from a misapprehension of the pipette method (2). The droplets containing the single organism may be made very small, and a mere film of liquid is present on the cover-glass, not a large rounded drop in the margins of which a second organism may lie concealed. The photomicrograph of a spore-bearing bacillus of *Bacillus tertius* (Fig. 1) illustrates the character of the droplet. This droplet has spread somewhat, owing to moisture condensed during the process of photography, and the small particles seen in it are probably due to the excess of vaseline used in the preparation of a specimen suitable for longer preservation during photography. In routine work the organism may be isolated in perfectly clear fluid and in droplets somewhat smaller than that shown in the illustration. After a series of organisms has been isolated, each droplet may be gone over with the oil immersion lens while the cover is on the isolation chamber, or, if the light there is insufficient, when temporarily removed to a shallow moist chamber. One cannot, of

course, exclude the possibility of the presence of any organism not visible to the oil immersion lens, but this limitation is common to all methods of isolating living organisms. If desired, the organism may be washed and rewashed in sterile broth, a procedure useful in removing any growth substances brought over from the original culture, but no guarantee against the inclusion of an invisible living substance. One may observe the first stages of development of an organism in a small drop of broth or at the margin of an agar droplet. In Fig. 2 a young colony of *Bacillus sporogenes* is shown growing out from the original position of a spore at the margin of an agar droplet.

The isolated organisms may be immediately transferred to test-tubes or grown on the cover-glass until colonies have formed and these then transferred. In transferring the organisms immediately after isolation the isolating pipette is discarded, and by means of new pipettes—a fresh one for each organism—the organisms are separately transferred to test-tubes. Each new pipette is supplied with a small amount of sterile broth before use, preferably from recently boiled broth or semifluid agar. In taking up an organism it is important to make sure that it enters the pipette and does not remain clinging to the margin of the opening. This precaution is not absolutely necessary in the case of aerobes or of anaerobes which are transferred to a liquid which is subsequently freed from air, but when a transfer to deep agar or to broth protected by a layer of vaseline or oil is intended, an organism not well within the pipette may remain on the vaseline or at the surface of the agar. In order to provide sufficient liquid for washing the organism well back a very small drop of broth may be made with the pipette in the immediate vicinity of the isolated organism and a small amount of broth added to the drop containing the isolated spore or bacillus just before it is taken up. The organism is then usually seen to enter the mouth of the pipette, and the additional droplet of broth is taken up in order to wash it well back. Sometimes a little manipulation is required to free an organism from substances which tend to make it cling to the film of surface tension of the droplet. Most bacilli and spores still enclosed in the mother cell give little trouble in this respect. When the tip of the pipette is brought to the desired depth in the medium in the test-tube, enough broth should be discharged to insure the

exit of the organism, and the discharge should be stopped before air is introduced.

With bacilli of some species it is desirable to minimize their exposure to the air during isolation. In this case it is well to introduce the isolating pipette directly into the culture from which isolations are to be made. The pipette is adjusted in the holder, a few isolations are made, and the organisms are transferred to an anaerobic environment as quickly as new pipettes can be prepared. Exposure to the air can be limited to 4 or 5 minutes, and the time of exposure can be fairly accurately measured. When pipettes for transferring the isolated organism are made in advance, or when two pipettes are used simultaneously, one for isolating and the other for taking up the organism, the time of exposure to air may be still further limited. No diluting fluid is needed in making isolations if bacilli are taken from a young culture just becoming cloudy.

Seeding Material.

In this work the aim has been to purify cultures isolated by the usual means. It might be possible to use the pipette method in isolating directly from the original source in cases in which the spores of anaerobes predominate and a considerable proportion of them are viable; but, in any case, it would be more convenient to make a preliminary isolation by one of the ordinary means. Spores for seeding were usually taken at maturity or within a few days afterwards, but in some instances spores from cultures 2 to 4 months old were sown and a fair proportion of positive results was obtained. In order to obtain proper bacilli for isolation, media were fairly heavily seeded, and the isolations made at the time when the first cloudiness appeared, usually within 4 or 5 hours. At this period nearly all the bacilli are living, and the longer elements or filaments which are then usually present are more easily isolated and possibly give a better chance of growth. In a few cases positive results were obtained with bacilli from cultures 24 hours old or more. In this paper the term "single bacillus" is made to include, in some cases, short filaments which may have been composed of several united bacilli.

Anaerobiosis.

In part of our series (Tables I and II, columns marked*) organisms were transferred to a fluid medium and the air was subsequently removed by means of an air pump. In the majority of cases boiling alone without vacuum was relied on to remove oxygen, and with

TABLE I.

Growth of Single Bacilli in Test-Tubes.

Species.	Medium and proportion of positives.													Total, all media.
	Glucose broth.	Serum glucose broth.	Semisolid glucose agar.	Semisolid serum glucose agar.	Semisolid serum agar.	Firm glucose agar.	Firm Veillon agar.	Liver peptone agar.	Minced brain.	Liver peptone water under vaseline.	Liver peptone water in vacuum.*	Milk.*	Plain broth.*	
B. sporogenes	2/14					0/6								2/20
" *welchii*	10/25		0/3						0/1	0/7	3/6	1/3	0/1	14/46
" *tetani*	13/36		3/12				1/3				0/5			17/56
" *œdematis*	3/12													3/12
" *botulinus*	3/6						1/3				0/5			4/14
B. of Ghon Sachs	0/16		1/7			0/1		0/6		1/6				2/36
B. aerofœtidus	1/3	0/1	1/2								0/23			2/29
" *putrificus*	0/2	1/2		1/3										2/7
" *bellonensis*	0/3	0/3	1/2	0/3										1/11
" *tertius*	0/1	2/3	0/2	0/2	3/3									5/11
" *fallax*	0/8			0/2	1/2									1/12
" *œdematiens*	2/3	0/2	1/1											3/6
" *bifermentans*	1/12	0/1	0/1	0/1	0/4					0/1				1/20
" *histolyticus*	1/1			1/3						2/2				4/6
Vibrion septique	0/47	0/8	1/37	0/12	0/7					0/3				1/114
Total, all species.	36/189	3/20	8/67	2/26	4/16	0/7	2/6	0/6	0/1	3/19	3/39	1/3	0/1	62/400

* Air removed after inoculation by means of an air pump.

fluid media access of air was prevented by a layer of vaseline 1.5 or 2 cm. thick. Immediately before inoculation the upper part of the vaseline layer was liquefied by gentle heat. Both firm and semisolid agar was used without any layer of vaseline or other protecting substance, and the isolated organism was introduced deep into the medium (Fig. 3). Except with serum-containing media, tubes were, as a routine, boiled and quickly cooled before use.

A simple method of anaerobiosis was found adequate for growing spores in hanging drops. A shallow moist chamber about 45 mm. long, 25 mm. broad, and 2 mm. deep, inside measurement, is made by cementing strips of glass to a large slide. Organisms are isolated in an area of about 3 by 1 cm. in the center of a large cover-glass. The isolated spores are arranged about 1.5 or 2 mm. apart, and enough medium is added after isolation to provide each spore with a droplet

TABLE II.

Growth of Single Spores in Test-Tubes.

Species.	Medium and proportion of positives.													Total, all media.
	Glucose broth.	Serum glucose broth.	Semisolid glucose agar.	Semisolid serum glucose agar.	Semisolid serum agar.	Firm glucose agar.	Firm Veillon agar.	Liver peptone water under vaseline.	Liver peptone water in vacuum.*	Milk.*	Plain broth.*	Egg cube.*	Meat.*	
B. sporogenes	11/16	2/5		3/6		2/5		5/6		1/1	1/2	1/1		26/42
" *tetani*	7/17		3/6	2/6			0/5		0/14					12/48
" *botulinus*							0/3		1/12					1/15
B. of Ghon Sachs	3/3		1/3											4/6
B. putrificus				4/5						1/1	1/1	1/1	1/1	8/9
" *bellonensis*		0/2	0/5	4/5	0/3				2/6					6/21
" *tertius*				10/12	1/1				1/7					12/20
" *œdematiens*				4/5					3/7					7/12
" *bifermentans*			3/3	4/6					1/3					8/12
" *histolyticus*									2/11		0/2			2/13
Vibrion septique			1/1	3/3			0/4		3/5					7/13
Total, all species.	21/36	2/7	8/18	34/48	1/4	2/5	0/12	5/6	13/65	2/2	2/5	2/2	1/1	93/211

* Air removed after inoculation by means of an air pump.

about 1 mm. in diameter, although this quantity may be made to vary, as desired. Larger drops sown with many organisms are also made for comparison. After the organisms have been isolated and supplied with medium, the under surface of the cover-glass, with the exception of the area in the center occupied by the isolations, is covered with soft glucose agar taken from the surface of a culture of *Bacillus pyocyaneus* about 4 or 5 hours old. This culture should contain many actively growing organisms, and a little fresh medium

may be added to it just before spreading. The area in the center of the cover may be enclosed in a ring of soft paraffin in order to prevent any danger of the spread of the *pyocyaneus* to the isolated anaerobes. The bottom of the moist chamber is supplied with a similar layer of *pyocyaneus* culture. The entire surface of the bottom may be covered, but better light is afforded for observation and there is less danger of condensation of an excess of moisture on the cover-glass if an area at the center of the bottom is left free. The cover is immediately sealed on the moist chamber by means of vaseline, and the chamber is enclosed in a Petri dish before it is placed in the incubator.

Pyrogallic acid and potassium hydroxide, so arranged that they may be mixed in the bottom of the moist chamber after the cover has been sealed on, were used alone and in connection with the *pyocyaneus* culture, but appeared to offer no advantage.

Preparations growing on the cover may be examined at any stage of growth and returned to the incubator without disturbing the cover-glass. Moist chambers taken out of the incubator should not be exposed to room temperature while still warm, since the cover-glass cools more rapidly than the slide and may accumulate an oversupply of moisture of condensation. It is best, then, to allow the preparations to attain room temperature before removing them from the Petri dish.

Media.

The media employed are shown in Tables I and II. Glucose media contained for the most part 0.5 per cent of glucose and were adjusted to a pH of approximately 7.4. Serum media contained 3 to 5 per cent of unheated rabbit serum, or, in a few instances, horse serum. The liver peptone water consisted of Dunham's peptone without salt adjusted to a pH of 7.8. The media were tubed, and to each tube a small piece of liver was added. It was then autoclaved, and after autoclaving the reaction was found to be about pH 7.4. Semi-solid agar media were made soft enough to be easily drawn into a fine pointed pipette while cold. As a routine, test-tubes of narrow diameter were used, and 10 cc. of media supplied to each tube.

Proportion of Positive Results Obtained from One-Cell Sowings in Test-Tubes.

Table I shows the number sown and proportion of positives of single bacilli sown into various media in test-tubes, and Table II gives similar data for spores. The denominators of the fractions in these and all subsequent tables give the number sown; the numerators, the number positive. Fifteen species, or possible species, are included. Questions as to the possible identity of certain species, as the bacillus of Ghon Sachs and *vibrion septique*, need not be discussed here. There were four strains of *Bacillus tetani*, one of them a Type III, two strains of *Bacillus botulinus*, and two of *Bacillus welchii*, one of which had been recently isolated from feces. For the most part the various strains had long been grown on nutrient media. In all strains of all species positive results were obtained either from single spores or from single bacilli, and in a majority of species from both. In the aggregate, 211 sowings of spores gave 44.1 per cent positive, while 400 sowings of bacilli gave only 15.5 per cent positive. The poorer showing of the sowings of bacilli is mainly due to the large number of negatives occurring in three species, *Bacillus aerofœtidus*, the bacillus of Ghon Sachs, and *vibrion septique*. The last two named gave positives with single spore sowings on the first trial.

As a routine, isolations were done in batches of five or six transfers. In a majority of species the first trial gave at least one positive among the first four tubes inoculated. In nearly 70 per cent of the thirty-six spore groups positives were obtained in at least one of the first four tubes inoculated. Fourteen spore groups, including nine species and ten strains, were sown into semisolid glucose agar, part with and part without serum. Of these, thirteen gave positives in at least one of the first four tubes inoculated. To isolate six spores or bacilli and transfer them to test-tubes requires, as a rule, less than 1 hour. These data give a better idea of the practicability of the method and the probabilities of success than do the percentages given in the tables, which include all experiments, tentative and otherwise.

Hardly enough experiments were made with all the different media to offer a fair basis for estimating their comparative value. The various glucose media served about equally well for bacilli, and semi-

solid serum glucose agar gave the best results for spores. Semisolid agar media not only give a relatively high proportion of positives, but offer the most convenient form for one-cell inoculations, since they do not need to be liquefied for inoculation and require no protective layer of vaseline or oil. Further, if positives are examined early one may often observe the formation of a single colony at the point where the organism was discharged. Certain advantages of semisolid media for routine work with anaerobes have been described by Lignières (3).

Bacteria in positive cultures in all media were examined microscopically, gas formation and other characteristics noted, and transfers made to broth or other media under aerobic conditions, in order to detect possible contaminations with air organisms. Such contaminations during the manipulations occur so rarely and are so easily detected that they constitute no source of difficulty in the pipette method.

Growth of Isolated Organisms in Hanging Drops.

The method of anaerobiosis for preparations in hanging drops has been described. Single spores from eight species were successfully grown. The media used were chiefly semisolid glucose agar, with and without serum, and serum glucose broth. The species tested and the proportion positive were as follows: *Bacillus tetani*, 17/57; bacillus of Ghon Sachs, 1/7; *Bacillus sporogenes*, 42/50; *Bacillus putrificus*, 1/8; *Bacillus bellonensis*, 11/24; *Bacillus œdematiens*, 4/12; *Bacillus tertius*, 10/19; *Bacillus bifermentans*, 1/29. Total, 87/206, or 42.2 per cent.

In some batches of all species single spores from the same source were sown at the same time into about 10 cc. of a similar medium in test-tubes. The test-tubes gave a total of 55.4 per cent positives out of 65 sown, while the corresponding hanging drops gave 38.2 per cent positives out of 102 sown.

The hanging drop method has certain advantages; isolations are quickly made—$\frac{3}{4}$ hour or less suffices for a series of ten isolations—and only two pipettes are required for a whole series, one for isolation and one for supplying additional media to the droplets. Early growth, variability, motility, and spore formation may be conveniently observed.

With species which readily form spores, it would probably save time in isolation to sow spores in hanging drops in preference to the direct transfer to test-tubes. The strains which fail to grow on the cover-glass could be reserved for the other method. Lag occurs in the hanging drop as in test-tubes, and preparations should not be discarded if no growth occurs on the 1st day. When colonies have formed in the hanging drop they may be easily transferred to the test-tube. In most species spores formed readily in the hanging drop, and these spores have afforded convenient material for further isolations.

The hanging drop method has not proved successful for growing isolated bacilli, although a large number of bacilli sown in one drop will often grow. A number of attempts to grow young isolated bacilli of several different species gave uniformly negative results, possibly because of too long exposure to air before sufficient anaerobiosis was attained.

Growth of Spores of Bacillus tetani in Homologous Serum.

The growth in hanging drops of single tetanus spores in a medium containing tetanus antitoxin was compared with similar sowings in a control medium containing no antitoxin. Both media consisted of semisolid glucose agar containing 3 per cent horse serum. The antitoxin serum contained approximately 325 units per cc., and the tetanus spores were taken from the strain used in immunization. Growth occurred in three out of ten sowings in the antitoxin medium, and in two out of ten controls. In a series sown in serum glucose broth the antitoxin medium gave one out of four positive, and the control one out of five. Spore formation and the quantity and character of the growth were similar in the two media, except that in the antitoxin medium bacilli showed a greater tendency to adhere in chains. Sowings of single spores in quantities of 10 cc. of the antitoxin medium gave two positive out of three sown. One of these positives is shown in Fig. 3.

Effect of Exposure to Air on Isolated Bacilli and Spores.

In the course of routine work the period of exposure to air was noted in many groups of experiments, and the proportions of positives occurring after various periods of exposure are shown in Table III. Only batches in which at least one positive occurred and only those in which young bacilli were sown are included in this table. The bacteria were exposed to the air in the very small droplets of the

TABLE III.

Effect of Exposure to Air on Young Bacilli.

Species.	Length of time exposed to air and proportion of positives.									Total, all periods.
	4–6 min.	7–10 min.	11–17 min.	18–23 min.	24–30 min.	31–36 min.	37–43 min.	44–60 min.	61–72 min.	
B. welchii	3/3	1/2	3/5	1/3	0/4	0/2	0/2			8/21
" *tetani*	3/7	3/4	3/7	3/10	1/4	1/5	1/3	1/7	0/1	16/48
" *œdematis*	2/2	1/1		0/3	0/1	0/2	0/2	0/1		3/12
" *botulinus*	1/1	0/1	1/1	0/2	1/1					3/6
" *sporogenes*	0/1	1/1	0/1	0/1	1/1	0/1				2/6
B. of Ghon Sachs	2/2	0/1	0/1	0/1	0/2					2/7
B. aerofœtidus	1/3	1/2	0/1							2/6
" *putrificus*	2/3	0/3	0/1							2/7
" *bi onensis*	1/3	0/2								1/5
" *tertius*	2/2	3/3	0/1							5/6
" *fallax*	1/2	0/2	0/2							1/6
" *œdematiens*	1/2	1/2	1/2							3/6
" *vifermentans*	1/3	0/3	0/2							1/8
" *histolyticus*	2/2	2/2	0/2							4/6
Total, all species	22/36	13/29	8/26	4/20	3/13	1/10	1/7	1/8	0/1	53/150
Percentage positive	61.1	44.8	30.8	20.0	23.1	10.0	14.3	12.5	0	35.3

liquid, usually glucose broth, in which they had been grown, and the period of exposure is reckoned from the time when a bacillus issued from the isolating pipette until it was deposited in the test-tube under anaerobic conditions. To the time of full exposure on the cover-glass one should add, theoretically, the time necessary for the reduction of any free oxygen introduced with the organism into the test-tube. However, the time necessary to attain sufficient anaerobiosis for growth must be short, since a very tiny amount of liquid is brought into 10 cc. of medium.

It appears from Table III that, on the whole, the probability of growth of young bacilli decreases with the increase in the time of exposure to the air, and that different species vary somewhat in sensitiveness to oxygen, as far as can be judged from the comparatively small numbers under each.

The results obtained with *vibrion septique* are not included in this table, since a different method of isolation was used for the batch in which a positive was obtained. Nearly 100 trials with sowings of young bacilli of *vibrion septique* were made by the usual method without success. The organisms were living when isolated, since in many cases the bacillus was distinctly motile, although motility ceased after an exposure to the air of $\frac{1}{2}$ or $\frac{3}{4}$ minute. A great variety of media was employed. The addition to glucose broth of salicin, of unheated pieces of rabbit kidney, and of broth from filtered young cultures of *vibrion septique* and of *Bacillus welchii* gave no success. It hardly seems likely that the medium was at fault, since single spores of this species grew readily in semisolid serum glucose agar—in one series of four all were positive. Furthermore, larger sowings of young bacilli grew rapidly in every medium tested. The time of exposure to air was cut down to $1\frac{1}{4}$ to 2 minutes by the employment of transfer pipettes prepared in advance and by the simultaneous use of two pipettes, but no growth was obtained. Sowings of ten or fifteen bacilli after an exposure to the air of 4 to 6 minutes also gave negative results.

Finally, a series of isolations was made under sterile paraffin oil spread on the cover-glass. The first oil series gave one positive out of seven sown. The organism which grew was motile when transferred. A second paraffin oil series of eight transfers gave all negatives, and all of a series of fourteen isolated under vaseline or vaseline diluted with paraffin oil were also negative. Motility persisted somewhat longer under vaseline than in similar droplets exposed to the air. In small hanging drops made in an atmosphere from which oxygen had been removed by pyrogallic acid and potassium hydroxide motility persisted for an hour or more.

It seems probable that young bacilli of *vibrion septique* are especially sensitive to free oxygen, and that this sensitiveness explains in large part the negative results obtained after the transfer of single bacilli.

Our experiments tend to confirm those of Bachmann (4), who found the vegetative forms of certain anaerobes very sensitive to an exposure to air of only 10 minutes plus the time required to obtain full anaerobiosis subsequently. In Bachmann's experiments bacilli were exposed to the air in agar plates, and the plates were subsequently brought under anaerobic conditions in which organisms remaining viable might form colonies.

In our experiments spores showed no reduction in the proportion of positives as the result of exposure to air during periods as great as 85 minutes at least. In 61 one-spore sowings, in which the time of exposure to air was noted, the periods of exposure and the proportion of positives were as follows: 12 to 17 minutes, 2/3; 18 to 23 minutes, 2/6; 24 to 30 minutes, 6/9; 31 to 36 minutes, 6/8; 37 to 43 minutes, 5/9; 44 to 60 minutes, 6/10; 61 to 72 minutes, 4/10; 73 to 85 minutes, 3/6. Total for all periods, 34/61. These results represent the aggregate of six species and include only batches in which at least one positive occurred.

The effect of exposure to air on anaerobes was not made the subject of a special study in our experiments, but only as incidental to the technique of isolation. It would appear that in the isolation of bacilli one should minimize the exposure to air as far as possible, while the viability of spores is not appreciably affected by an exposure of an hour or more.

Lag.

In a great majority of the experiments the inoculated test-tubes were not enclosed in an anaerobic jar, and the time of first appearance of growth could be approximately noted. In all positives occurring in tubes in which bacilli were sown, growth appeared on the day following inoculation. The first appearance of cloudiness in broth or of a colony in agar varied approximately from 16 to 21 hours after sowing. Growth from single spore sowings showed a marked tendency to lag. The amount of lag by days is shown in Table IV. A day was reckoned to the time of the last observation, usually about 5 p.m. If growth appeared on the 1st day, it was usually later than in sowings of single bacilli of the same species. It is seen from Table IV that about 37 per cent of the positives appeared on the 2nd day or later,

thus showing a lag of a day or more. The condition of the spores sown is doubtless an important factor. This fact is illustrated by the results with the washed spores of *Bacillus tertius*. Spores were twice washed by centrifugation in normal salt solution, and two of the three positives lagged 3 days or more. Three grew out of a series of five sown. Unwashed spores from the same source gave all positives, and all grew without lag. In addition to the washing the spores were kept several hours in salt solution at refrigerator temperature.

TABLE IV.

Time of Appearance of Growth from Single Spores Sown in Various Media in Test-Tubes.

Species.	Total No. of positives.	Day following inoculation on which growth appeared and incidence of positives.						
		1	2	3	4	5	6	8
B. sporogenes	20	6	12	2				
" *tetani*	12	9	2		1			
" *putrificus*	6	4	2					
B. of Ghon Sachs	4	4						
B. bellonensis	4				2		1	1
" *œdematiens*	4	4						
" *tertius* (unwashed spores)	8	8						
" " (washed ")	3	1			1	1		
" *bifermentans*	7	7						
Vibrion septique	4	2		1	1			
Total	72	45	16	3	5	1	1	1

The marked tendency to latency observed in the growth of the spores of some species illustrates one source of difficulty in separating these from other species by plate methods.

Variability in Morphology of Bacillus sporogenes.

The method employed in isolation is available for a study of variability in anaerobes as in aerobes, as is shown by the following experiment. Four spores from a culture of one-cell origin were isolated and sown in hanging drops. Of the four colonies formed, one showed a marked tendency to the formation of filaments, these appearing in the form of a fine network. Six spores from the filamentous colony

were isolated in a new series of hanging drops, and all showed the same filamentous tendency to a greater or less degree as compared with a series of controls from a non-filamentous source. In further series on a different medium the filamentous strain reverted to normal. A long filament isolated and transferred directly to a test-tube gave the same type of growth as controls.

DISCUSSION.

It is a matter of common knowledge that large sowings of bacteria into a new medium give a better chance of growth than small sowings. Large sowings offer a larger assortment of individuals, some of which may be more vigorous or otherwise better capable of adapting themselves to new conditions, and, furthermore, any growth products carried over with the bacteria may favorably modify the new environment. When a single cell is sown the change in environment is more complete, and unless the new environment is identical with the old a process of adaptation may be necessary. In the experience of the writer, single aerobes have often given 100 per cent of positives when sown into as much as 10 cc. or more of new medium favorable to the species sown.

The importance of employing a suitable medium is shown by experiments on Pneumococcus Type I. Two batches of plain broth made during successive weeks and apparently similar in constituents and reaction were compared. Six tubes of Batch 1 and six of Batch 2 were alternately sown with pneumococcus taken at the height of growth. Tubes of Batch 1 received each one pair of pneumococcus, and those of Batch 2 received each one to four pairs. All the tubes of Batch 1 showed abundant growth which proved to be pure culture of pneumococcus, while all the tubes of Batch 2 remained sterile. All the tubes contained 10 cc. of medium. A flask containing 75 cc. of Broth 1 also gave an abundant growth with a sowing of one pair. Large sowings in Broth 2 grew readily.

When single anaerobes are sown, the lack of proper conditions of anaerobiosis may offer more obstacles to growth than is the case when larger sowings are employed. Burri and Kürsteiner (5) have shown that the growth of *Bacillus putrificus* initiated under strict anaerobic

conditions may continue with increased activity under conditions of aerobiosis unfavorable to the beginning of growth.

In our experiments 100 per cent of positives were obtained with certain lots of five or six tubes each in which single spores of *Bacillus tertius*, *Bacillus sporogenes*, and *Bacillus bifermentans* were sown. Single spores from a 48 hour culture of *Bacillus subtilis* gave all positives in six tubes of glucose broth with a vaseline layer. The medium was the same as that used for anaerobes except that the free oxygen was not expelled by heat. These results would indicate that the technique of the transfer of the single organism is not at fault, and that failures to grow should be ascribed to a lack of viability of the organism or to its failure to adapt itself to a new environment. The viability of anaerobic bacilli is affected by exposure to air, a fact which explains in part the small proportion of positives as compared with single bacilli or cocci of aerobes. The negatives in spore sowings can hardly be due to lack of proper conditions of anaerobiosis, since spores are little sensitive to short exposures to air, and abundant time is available for the reduction in the new medium of the small quantity of free oxygen introduced with the spore. The quantity of medium to which the spore is transferred can hardly be an important factor in itself, since spores sown in small hanging drops give a smaller proportion of positives than when sown into test-tubes. The quality of the medium is probably more often the determining factor, and if viable spores and a medium exactly suited to each species are employed, all, or practically all the spores might be expected to grow. The process by which an organism adapts itself to a new and less favorable medium is imperfectly known. Our experiments indicate that organisms apparently similar vary greatly in their power of adaptation.

SUMMARY.

The pipette method has proved a feasible method of obtaining one-cell pure cultures of anaerobes.

Both bacilli and spores may be used as seeding material, but spores give a much higher percentage of positives.

Boiling alone affords a sufficient degree of anaerobiosis to the medium for initiating one-cell growths, and semisolid agar is the most convenient form of medium.

Exposure to air during isolation apparently has no effect on the viability of spores of anaerobes, but young bacilli of some species suffer from a comparatively short exposure to free oxygen.

I wish to thank Colonel F. F. Russell, in charge of the Army Medical School at Washington, where a portion of this work was done, and Major Benjamin Jablons, recently in charge of the work on anaerobic bacteria in the Army Medical School, for many courtesies shown and for a large proportion of the cultures of anaerobes used in these experiments. I am also indebted to Dr. Charles Krumwiede, of the Research Laboratories of the Department of Health of the City of New York, for a quantity of tetanus antitoxin.

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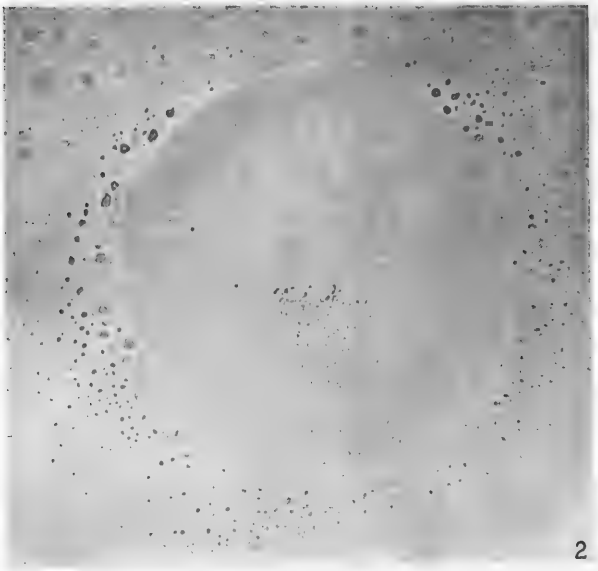
4. Bachmann, F., *Centr. Bakteriol., 2te Abt.*, 1912–13, xxxvi, 1.
5. Burri, R., and Kürsteiner, J., *Centr. Bakteriol., 2te Abt.*, 1908, xxi, 289.

EXPLANATION OF PLATE 23.

FIG. 1. Spore-bearing bacillus of *B. tertius* isolated in a hanging drop of broth. × 1,000.

FIG. 2. Colony of *B. sporogenes* grown from a single spore in a hanging drop of semisolid glucose agar. × 120.

FIG. 3. Colony of *B. tetani* grown from a single spore in glucose agar containing tetanus antitoxin. About natural size.



(Barber: Anaerobes.)

EXPERIMENTAL INOCULATION OF MALARIA BY MEANS OF ANOPHELES LUDLOWI.*

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Doubt has existed in the minds of certain sanitarians in Farther India as to the part played by *Anopheles ludlowi* as a transmitter of the malaria on the littoral of the Peninsula of Malacca and Indonesia.

Christophers in a report of his investigation[1] of malaria in the Andaman Islands in 1912 records the discovery of malarial zygotes in two out of 53 naturally infected wild specimens of *Anopheles ludlowi* and found that the relation between the distribution of the species and that of malaria was most exact.

Watson,[2] however, has expressed the opinion that, from what he has seen in the Federated Malay States, *Anopheles ludlowi* can exist without producing malaria and that malaria will disappear from a place when operations are undertaken which abolish only *Anopheles umbrosus* yet which leave very large numbers of *Anopheles ludlowi*.

Strickland in his report to the Principal Medical Officer of the Federated Malay States, on the cause of the outbreak of malaria at Morib, Federated Malay States, concluded from epidemiological findings that "*Anopheles ludlowi* was causing most of the trouble."

This disparity of opinion among British workers as to the part played by *Anopheles ludlowi* in transmitting malaria is largely if not wholly due to the presence of other species known to be hospitable hosts for malaria, notably *Anopheles umbrosus*. At Port Swettenham, for example, I collected at and near the Rest House *Anopheles ludlowi*, *Anopheles kochi*, *Anopheles umbrosus*, *Anopheles rossi*, *Anopheles*

* This work was done under the auspices of the British Colonial Office and with the Support of the Government of the Federated Malay States and The Rockefeller Foundation.

[1] Christophers, S. R., Malaria in the Andamans, *Sc. Mem. Med. and San. Dept., Gov. India, No. 56*, 1912.

[2] Watson, M., Rural sanitation in the Tropics, London, 1915, 83.

indefinitus, *Anopheles tessellatus*, *Anopheles barbirostris*, *Anopheles albirostris*, and *Anopheles sinensis*. Barber succeeded in a more exhaustive search in taking one specimen of *Anopheles karwari*. He has also succeeded in infecting all the species mentioned except *Anopheles tessellatus*. Hence the occasion for the doubt that existed in the minds of the local epidemiologists as to the importance of *Anopheles ludlowi*.

In 1916 while studying the cause of anemia among the natives of Java I found epidemiological evidence that the malaria in certain villages, or dessas, near Cheribon on the north shore of Java was due to *Anopheles ludlowi*. In the dessa of Gebongelir there was severe malaria, the splenic index at the time was 97 (101 persons examined), and the endemic index, or parasite rate, was 29 per cent. Mosquitoes were collected daily from the houses of the villagers for dissection. The species chiefly encountered was *Anopheles ludlowi*. Six of the 58 specimens dissected contained malarial zygotes. There were a good many breeding places in the fish ampangs, or reservoirs, near by, and from larvæ collected thereabouts *Anopheles ludlowi* was the only species that emerged. At the same time 89 *Anopheles rossi* Giles from the adjoining village of Gebongoedik were examined for sporozoites and 32 for zygotes with negative result. The weight of the evidence here is that *Anopheles ludlowi* was the carrier of malaria in the village.

It is this epidemiological evidence that is of most value to the sanitarian, for it is possible for *Anopheles* hospitable to malaria to bite malarial patients and become infected and to have sporozoites in their salivary glands, yet if the mosquitoes feed on animals as well as man the infectious sporozoites may often be dissipated on the latter. This is a matter that has been overlooked in discussing the question of efficient agents for transmitting malaria.

Barber[3] has shown that most *Anopheles* in Malaya can be infected with malaria under experimental conditions. Yet I have taken *Anopheles umbrosus* and *tessellatus* which had been feeding on fowl blood for they were caught near hens' nests with nucleated red blood cells in their midguts, and I have also taken many specimens of *Anopheles umbrosus*, *Anopheles rossi*, and *Anopheles indefinitus* in cow barns.

[3] Barber, M. A., Malay *Anopheles* in transmission of malaria, *Philippine J. Sc., Section B*, 1918, xiii, 1.

In Brazil *Anopheles albimanus* feeds with avidity on horses and the latter animals are used as bait to catch the mosquitoes. It is evident that when a species of *Anopheles* known to be a carrier fails to take up an obligate parasitic relation with man and through feeding on animals becomes essentially only a facultative parasite, it cannot be regarded as a thoroughly efficient host for malaria no matter how easily it may become infected in the laboratory. It is the epidemiological evidence, therefore, in the last analysis which convicts a species and will determine the necessity for taking steps against it.

These observations in Java were made in August to October, 1916, at a time when I had already become thoroughly convinced of the importance of *Anopheles ludlowi* as a carrier of malaria in the littoral of Indonesia. The previous year, October to December, 1915, I had found that *Anopheles ludlowi* could be infected experimentally with ease. Out of a number of *Anopheles ludlowi* reared from larvæ taken at Port Swettenham, Federated Malay States, and fed on suitable patients that had sufficient gametes in their peripheral blood, 40 were dissected at intervals of 2 to 12 days after feeding. 20 were found to have zygotes in their midguts and 5 to have sporozoites in their salivary glands. 25 out of 40, therefore, had become infected, a percentage of 62. It was evident that not only could zygotes develop in the tissues of this species but sporozoites could reach the salivary glands and these in a sufficient number of mosquitoes to warrant the assumption that the species was a carrier of malaria. Barber[3] at the same time from a series of experiments came to the same conclusion.

Since then[4] studies on the relation of *Anopheles ludlowi* to malaria in Java and Sumatra have been published by Schueffner, Swellengrebel, Swellengrebel de Graaf, and native Javanese doctors. In these papers *Anopheles ludlowi* is shown conclusively to be a carrier of importance.

While engaged in examining the dissection of the salivary glands it appeared to me that the lumen of the gland ducts was very small, perhaps too small to permit the passage of sporozoites outward from the acini into the proboscis of the mosquito. If this were the case,

[4] Schueffner, W., Swellengrebel, N. H., and Swellengrebel de Graaf, J. M. H., Mededeelingen van den Burgerlijken Geneeskundigen Dienst in Nederlandsch-Indië, 1919, 1.

malarial plasmodia might readily enough develop in the species and sporozoites reach the salivary glands, yet the mosquitoes would be unable effectively to transmit malaria.

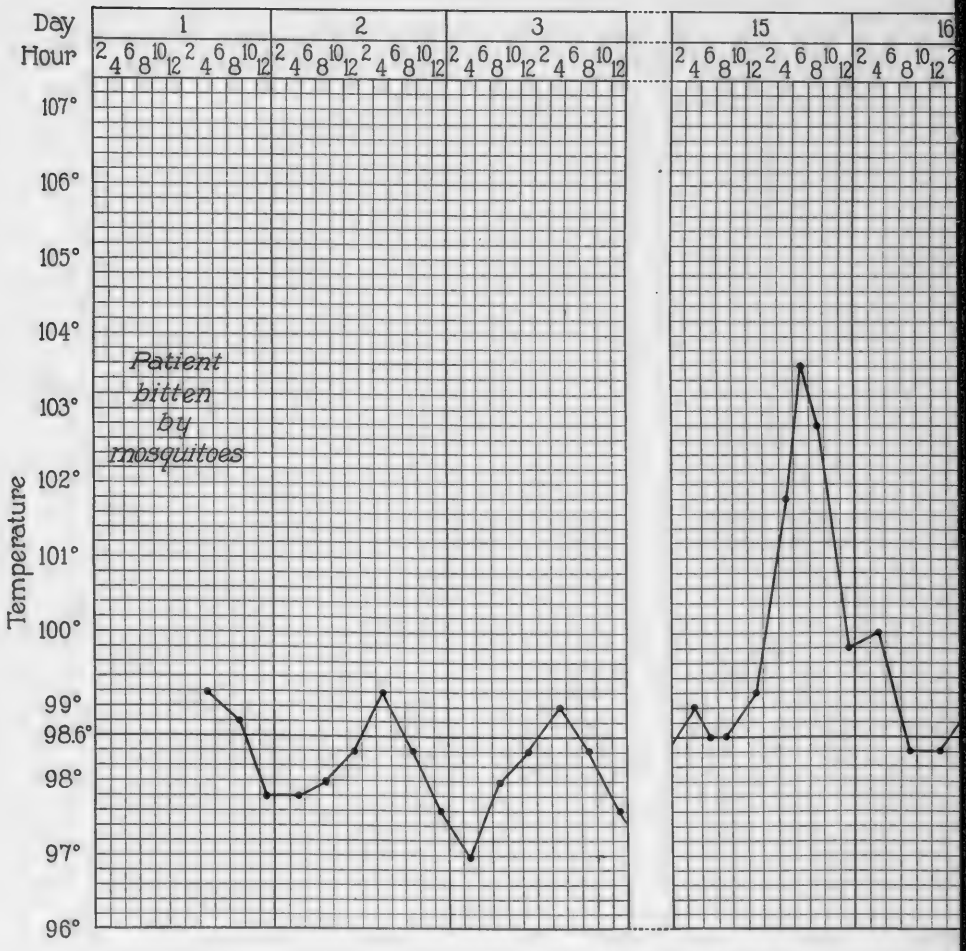
Additional antimalarial drainage operations at this time were being planned by the Malarial Advisory Board. It was of some importance for their engineer to know whether measures should be adopted against *Anopheles ludlowi* in addition to those projected against *Anopheles umbrosus* which breed in entirely different locations. For this as well as for other reasons it was decided to undertake inoculation experiments with *Anopheles ludlowi* on man.

EXPERIMENTAL.

Inoculation Experiments.

The experiments were planned to determine the following points: (1) Whether *Anopheles ludlowi* infected under experimental conditions serves to infect man with malaria and with what facility; (2) whether infected mosquitoes that have already bitten one person serve to infect others; (3) the period of incubation in man; (4) the rate of appearance of plasmodia in the inoculated person and how it corresponds with the appearance of fever; (5) what differences if any can be noted in the clinical picture of (*a*) a man being infected with malaria for the first time, (*b*) a man being reinfected with malaria after a long interval of years during which time he has been free from evidences of the disease, (*c*) a man being infected with subtertian malaria who is at the time suffering from tertian malaria; and (6) what type of febrile reaction is induced by a single inoculation of an unmixed strain of subtertian plasmodia (sporozoites).

Malaria is highly endemic in many places outside the cities, towns, and certain protected areas in the Peninsula of Malacca. Several attacks of malaria may be expected by every coolie during his period of residence in the country, particularly if he lives on a rubber estate. Consequently, when the nature of the test was explained to them, there was no difficulty in securing three men who volunteered for the experiment.



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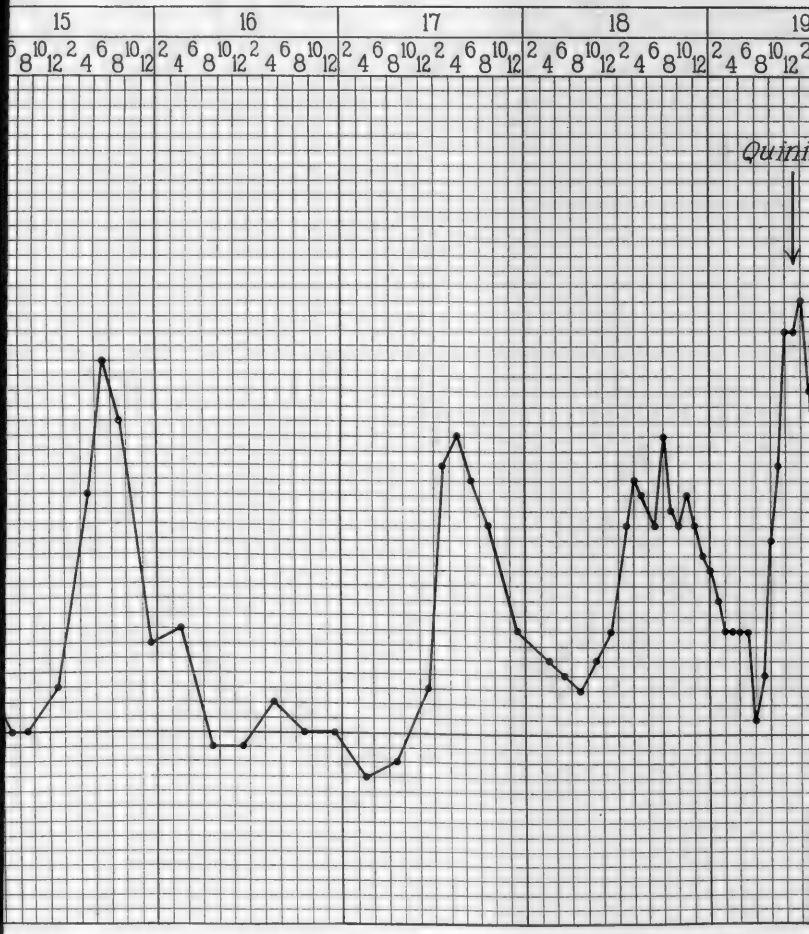

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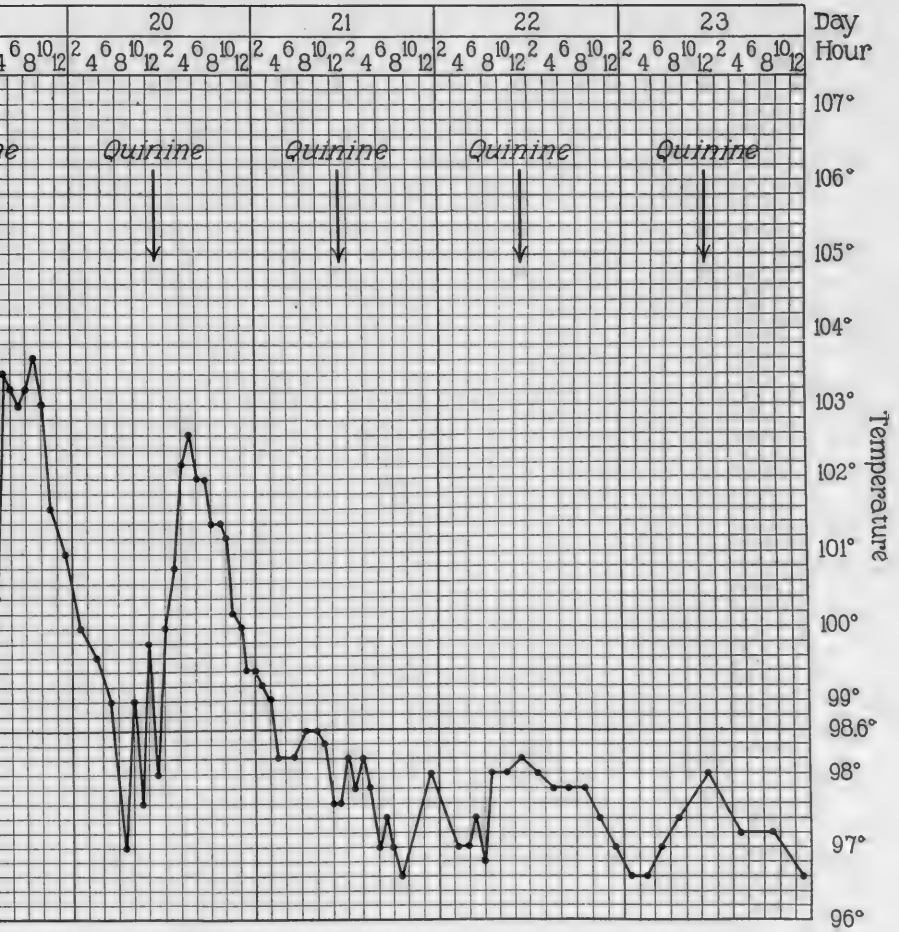
TEXT-FIG. 1. Temperature curve of Subject 1.



remained within normal limits.

Subject 1 (*Text-Fig. 1*).—L. K. Male, age 37 years; a Chinese mining coolie, resident 18 months in Malaya with a negative history for malaria. He was a patient in the surgical ward with a broken leg; otherwise normal and in good health. Spleen normal; blood negative for malarial plasmodia.

Dec. 1 to 3, 1915, p.m. Bitten by six mosquitoes. Dec. 8. Blood count: Red blood corpuscles 4,829,800; leucocytes 6,200; hemoglobin 85 per cent. Dec. 14. The first clinical note was made on this date when the patient complained of loss of appetite; his tongue was furred. 4 p.m. Temperature 99.4°F. Dec. 15. Pain in back and joints; face flushed; spleen which was normal became palpable below costal margin. 3.30 p.m. Rigors. 6 p.m. Temperature 103.6°F. 9 p.m. Subtertian rings were found in blood. Dec. 16. Sweated a good deal but feels well during the remission of fever. Subtertian rings in blood. Dec. 17, 1 p.m. Mild rigor. Spleen palpable below costal margin. 2 p.m. Temperature 102.2°; tongue coated; bad taste in mouth. Subtertian rings in blood. Dec. 18. Spleen palpable; tender. 4 p.m. Temperature 101.8°F. Subtertian rings in blood. Red blood corpuscles 5,100,000; hemoglobin 79 per cent. Dec. 19. Very ill; vomiting is troublesome. 12 m. Temperature 104°. Spleen 1 fingerbreadth below costal margin; tender. Very few subtertian rings in thin film of blood. Quinine administration begun. Quinine bihydrochloride 15 grains three times a day; retained about 30 grains only. Dec. 20. Very ill; vomited some quinine and all food. Subtertian rings in blood. Dec. 21, a.m. Better; vomiting less frequent; able to retain quinine 20 grains twice a day. Very few subtertian rings in blood. Dec. 22. Very weak; vomiting ceased; 10 grains of quinine three times a day. Blood negative. Dec. 23. No fever; a little better; spleen palpable below costal margin but diminished slightly in size; 10 grains of quinine three times a day. Dec. 24 and 25. Feels better; spleen just palpable; 10 grains of quinine twice a day. Dec. 26. Quinine discontinued. Jan. 1 to 11. There were at times epigastric pain, flushing of the face, and loss of appetite with slight elevations of temperature similar to those observed before the malarial infection. Jan. 17. Patient quite well. Jan. 28. Discharged.

In Subject 1 there was observed a diminution in body weight beginning about Dec. 10 before the onset of fever and lasting until about Jan. 17, 1916 (Table I). On Dec. 22, or the day after the crisis, the loss in weight amounted to 3 catties, or about 4 pounds. His physical strength as measured by the average of three squeezes with the hand grip dynamometer showed a corresponding diminution, dropping from 68.6 on Dec. 11 to 55 on Dec. 24, and returning slowly to 68.6, or normal, 1 month after the crisis.

This was a severe case of malaria with relatively few plasmodia and only moderate enlargement of the spleen.

Subject 2 (*Text-Fig. 2*).—S. Male, age 30 years; Tamil coolie, resident 18 months in Malaya. Has had several attacks of tertian ague with fever and rigor on alternate days during the past 6 months, and on Dec. 1, 1915, tertian plas-

modia of all ages were encountered in the peripheral blood. He did not complain of fever but an inspection of his chart shows quotidian oscillations reaching 99° or 99.5° daily. The spleen was not palpable. He was operated on for hemorrhoids Nov. 11.

Dec. 1. Bitten by six mosquitoes that had a few seconds before been interrupted while feeding on Subject 1. Dec. 8. Blood count: Red blood corpuscles 2,732,800; leucocytes 3,600; hemoglobin 60 per cent. The blood picture from Dec. 1 to 22 consisted in varying numbers of tertian plasmodia. Dec. 12. Tertian rosettes were seen; on the following 3 days there were quotidian rises of tempera-

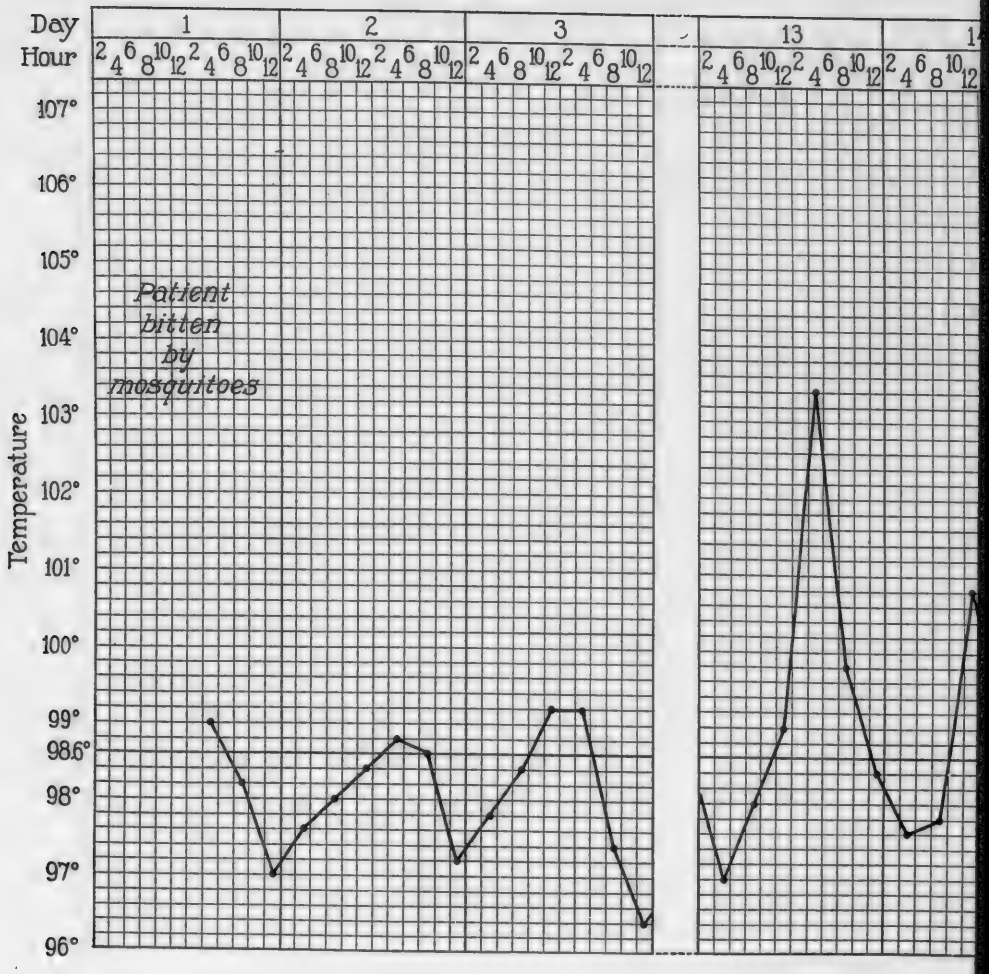
TABLE I.

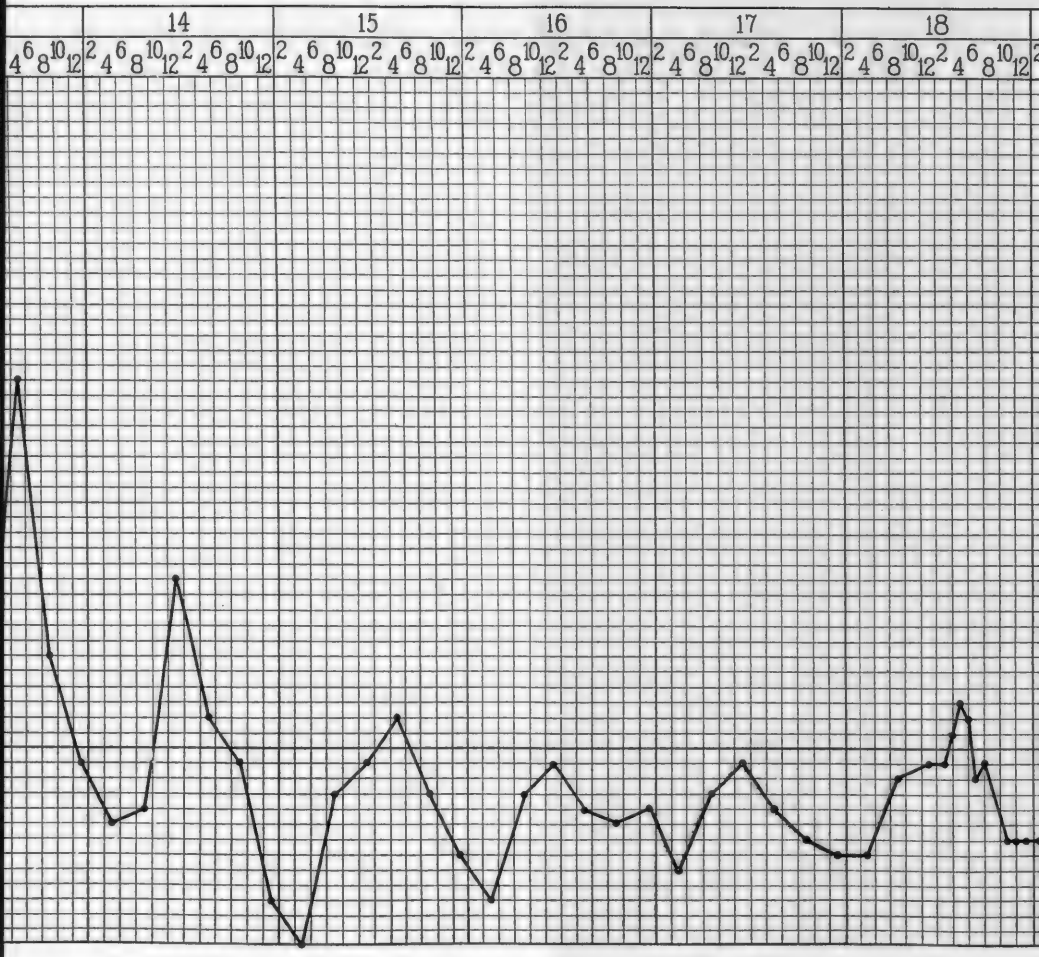
Body Weight and Strength (Hand Grip) of Subject 1.

Date.	Hand grip.	Weight in catties.*
1915		
Dec. 6		85
" 8	68.3	
" 10		84
" 11	68.6	
" 16	63	83
" 20	57.3	
" 22		82
" 24	55	
" 28	59	83
1916		
Jan. 1	62.3	
" 3		83
" 4	63.3	
" 9	63.3	
" 10		80
" 13	65	
" 17	65	85
" 20	68.6	85

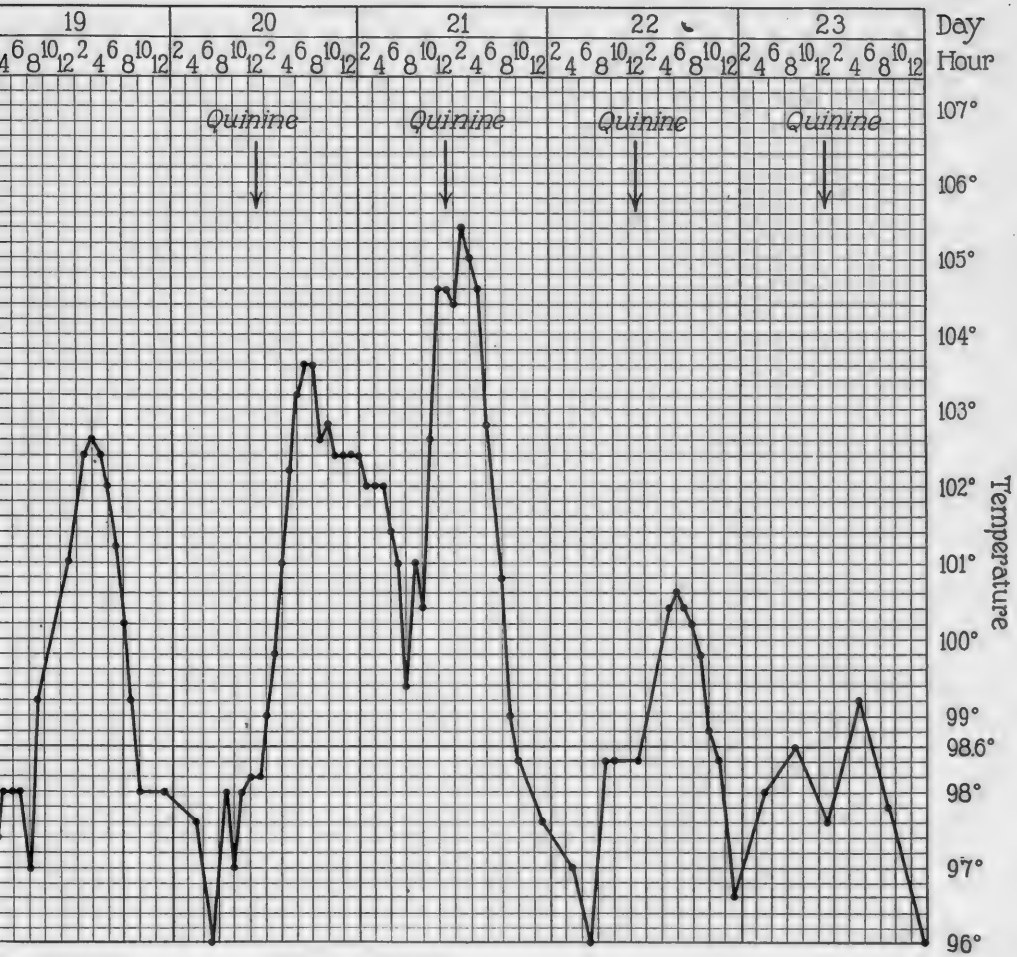
* A catty is the oriental weight equal to $1\frac{1}{3}$ pounds avoirdupois.

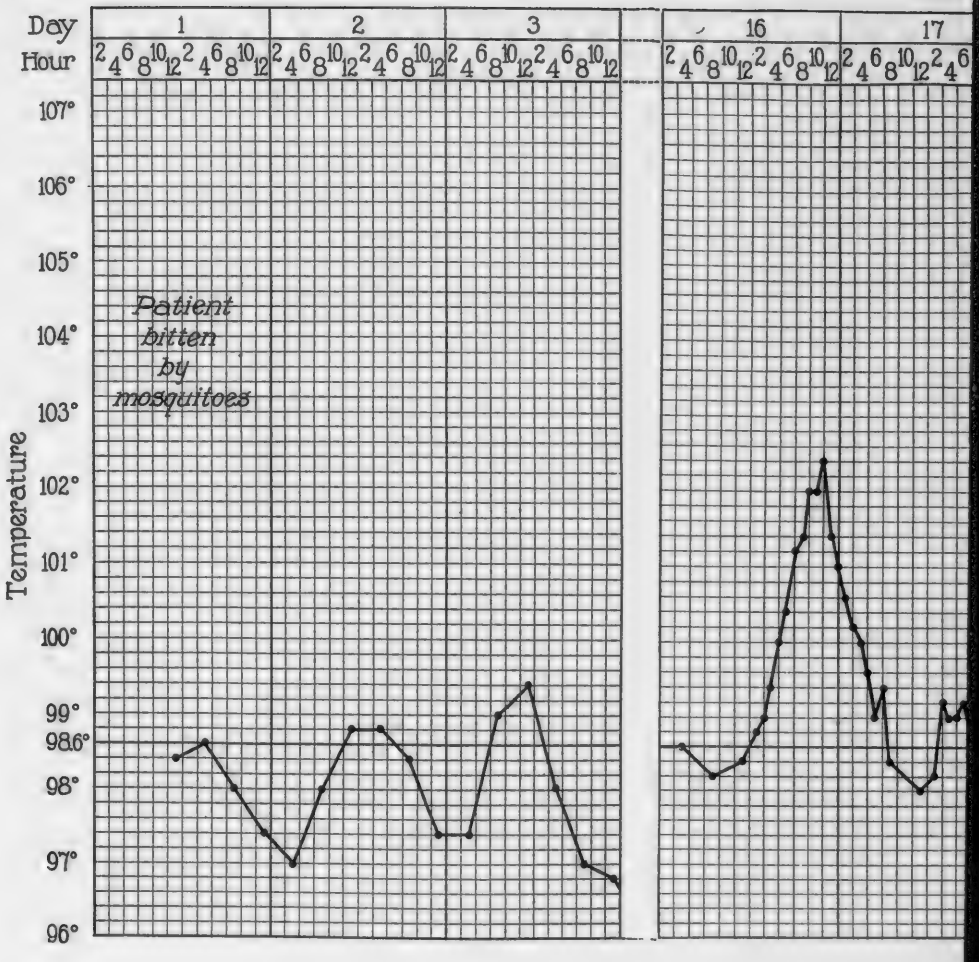
ture. Dec. 13 to 17. A few trophozoites resembling subtertian forms were seen. Dec. 18. Blood count: Red blood corpuscles 3,149,600; hemoglobin 55 per cent. Dec. 20. Subtertian rings definitely present in large numbers. On account of the ease with which some very young tertian trophozoites may be mistaken for subtertian merozoites one can never be absolutely certain of the identity of any given trophozoite. The specific identity of the forms seen on Dec. 13 and 17 cannot be asserted with absolute confidence. Dec. 21. Many subtertian rings were seen. Dec. 22. Fewer subtertian rings were seen. Dec. 23 to Jan 12, 1916. No trophozoites either tertian or subtertian were detected in thick films.

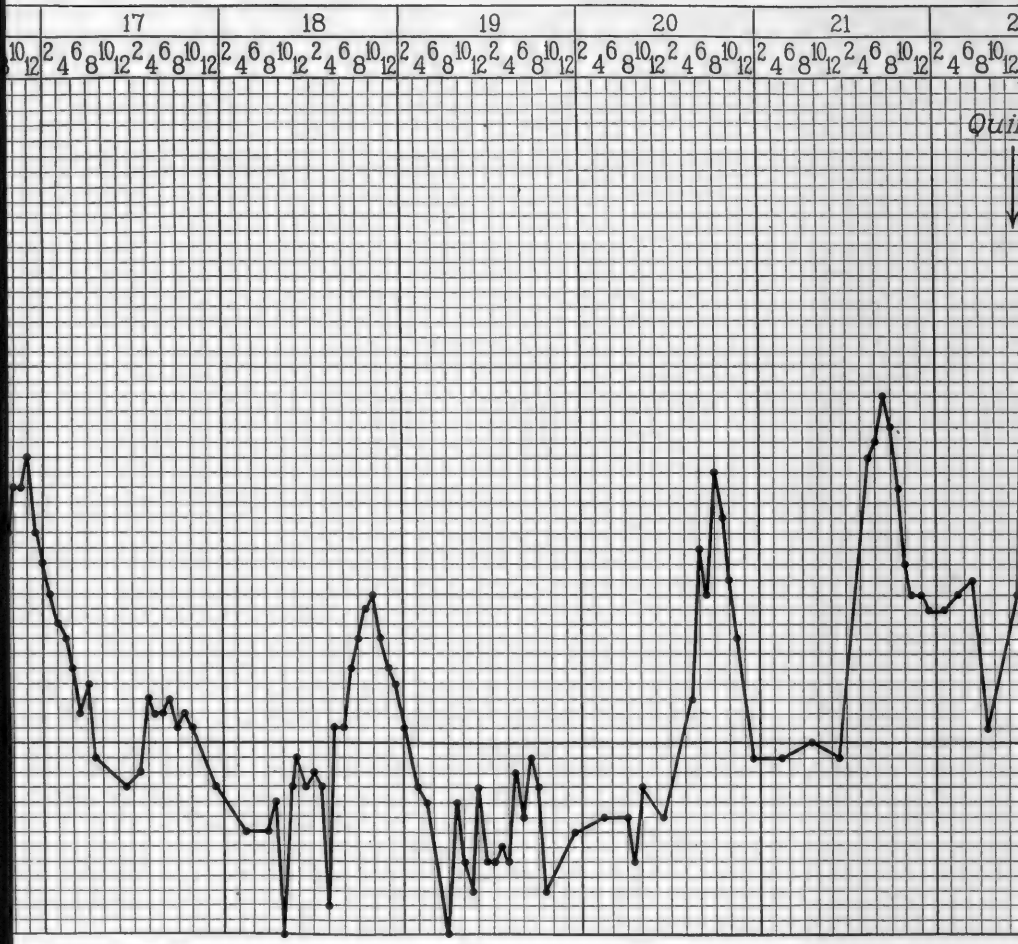

Day
Hour
1
2
3
13
14
2 4 6 8 10 12 2 4 6 8 10 12
Temperature
107°
106°
105°
104°
103°
102°
101°
100°
99°
98.6°
98°
97°
96°
Patient bitten by mosquitoes



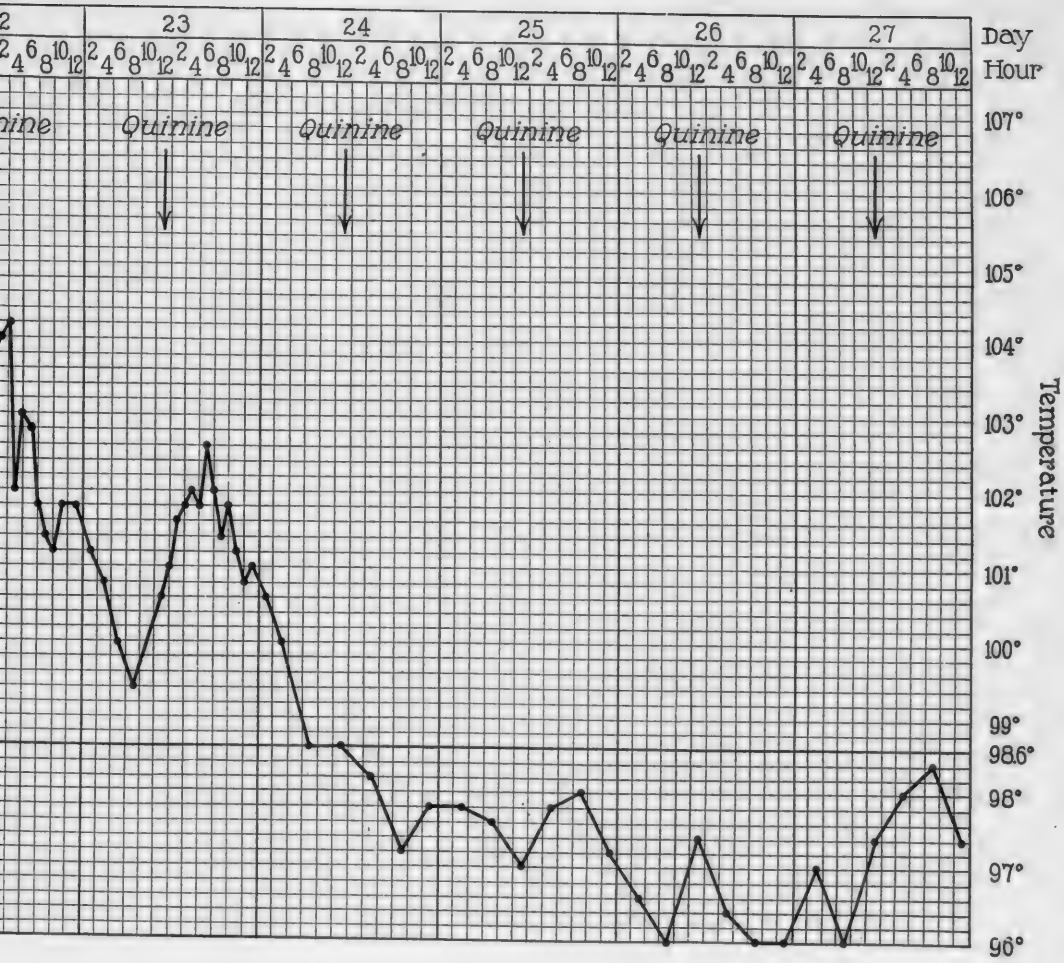
TEXT-FIG. 2. Temperature curve of Subject 2.


19
20
21
22
23
Day
Hour
Quinine
Quinine
Quinine
Quinine
107°
106°
105°
104°
103°
102°
101°
100°
99°
98.6°
98°
97°
96°
Temperature


Day
Hour
1
2
3
16
17
2 4 6 8 10 12 2 4 6 8 10 12
Temperature
107°
106°
105°
104°
103°
102°
101°
100°
99°
98.6°
98°
97°
96°
Patient bitten by mosquitoes



TEXT-FIG. 3. Temperature curve of Subject 3.


22
23
24
25
26
27
Day
Hour
2 4 6 8 10 12
Quinine
Quinine
Quinine
Quinine
Quinine
107°
106°
105°
104°
103°
102°
101°
100°
99°
98.6°
98°
97°
96°
Temperature

Clinical Notes.—Dec. 13. Temperature rose to 104.4°F. without rigor.[5] Apparently he was suffering no inconvenience from this tertian infection. The spleen was not enlarged. Dec. 19, 4 p.m. Temperature rose to 102.4°. Spleen not enlarged. Patient appeared normal. Dec. 20, 2 p.m. Temperature 99.8°. 6 p.m. Temperature 103.6°. Quinine bihydrochloride 10 grains three times a day. Dec. 21, 12 m. Temperature 104.6°. Quinine bihydrochloride 10 grains three times a day. Dec. 22. Patient was slightly inconvenienced by the infection; quinine bihydrochloride 40 grains; spleen not enlarged. Dec. 23 to 29. Quinine bihydrochloride 10 grains three times a day. Patient appeared quite normal, but there was a decided daily drop in temperature from 12 m. to 8 a.m. Dec. 30. Quinine discontinued. Dec. 31 to Jan. 7. Patient quite normal. Weight increased from 100 catties on Dec. 28 to 105 catties on Jan. 17. Jan. 22. 50 hookworms were expelled after a treatment with chenopodium.

This was a mild case of malaria as far as symptoms were concerned, but the number of plasmodia encountered was considerable. The febrile reaction, size of spleen, and multiplication of subtertian plasmodia were not in the slightest degree influenced by or interfered with by the preexisting tertian infection.

Subject 3 (*Text-Fig. 3*).—J. Male, age 45 years; a Tamilian hospital attendant, resident 26 years in Malaya. During the past 7 years he had been entirely free from attacks of malaria but previous to this he had suffered from malaria several times, the attacks lasting about 10 days. Was being treated for a compound fracture of ulna with necrosis of bone. Blood was negative for malaria. Spleen normal.

On Dec. 3, 1915, bitten by three mosquitoes and on Dec. 4 by two mosquitoes that had previously fed on Patients 1 and 2 on Dec. 1. Dec. 8. Blood count: Red blood corpuscles 3,680,000; leucocytes 7,600; hemoglobin 57 per cent. This anemia may have been related to the injury to his elbow. There was certainly no malaria and no ancylostomiasis. Dec. 17. Facial expression has changed; patient looks drowsy. Complains of pain in elbow. The spleen which had been negative is palpable below the ribs. Dec. 18. Appeared well. Very few subtertian rings in thick film of blood; thin film negative. Red blood corpuscles 3,949,600; hemoglobin 66 per cent. 8 p.m. Temperature 101.4°, with rigors. Dec. 19. Spleen 2 fingerbreadths below ribs and tender; fever and rigors previous night; not very ill. Subtertian rings in thick film of blood; thin film negative. Dec. 20. Fever and rigor. 8 p.m. Temperature 100.4°. Spleen unchanged;

[5] The occurrence of latent malaria without symptoms is far more common than is suspected. In Panama I found 10 per cent of 322 Spanish laborers to be harboring plasmodia in their blood. The blood specimens were taken at the mess hall and none of the men was ill or complaining at the time.

not very sick. Thin film of blood negative. Dec. 21. Marked remission; spleen unchanged. Few subtertian rings in thick film of blood. Dec. 22. Rigors and fever. 8 p.m. Temperature 101.8°. Spleen 2½ fingerbreadths below ribs; conjunctivæ injected; face flushed. 11 p.m. Sweating. Fewer subtertian rings than yesterday in thick film of blood. Dec. 23. Condition the same. 4 p.m. Temperature 102.4°. Fewer subtertian rings in thick blood film than day before. Dec. 24. Very ill and weak. 4 p.m. Temperature 102°. Spleen at level of umbilicus and tender; tongue coated. Dec. 24 to Jan. 11. Blood negative. 8 p.m. Quinine bihydrochloride 20 grains. Dec. 25. Very weak. Temperature 102°. Quinine bihydrochloride 15 grains three times a day. Dec. 26. Better; temperature normal; spleen 1 fingerbreadth below umbilicus; tender; no vomiting. Dec. 27. Quite well. Dec. 28. Quinine bihydrochloride 10 grains three times a day. Jan. 8, 1916. Quinine discontinued. Jan. 11. Spleen at level of umbilicus. Jan. 18. Spleen 2 fingerbreadths above level of umbilicus. Jan. 20. Discharged.

This was a case of malaria of ordinary degree of severity in which plasmodia were not very abundant but in which the splenic reaction was pronounced and was manifest before plasmodia appeared in the peripheral blood.

Method of Infecting Mosquitoes and Inoculating the Men.

The method was identical with that used in Panama[6] in 1908. Full grown larvæ of *Anopheles ludlowi* were collected at Port Swettenham and reared at Kuala Lumpur. The mosquitoes after emerging were confined in biting jars made of lantern chimneys. The air in the jars was kept as moist as possible and sugar was given when necessary. Both ends of the jars were securely covered with crinoline gauze through which the mosquitoes bit. The mosquitoes were at first applied daily to the skin of a gamete carrier in order to favor the infection of as many mosquitoes as possible and also to reduce the mortality from desiccation. The jar of mosquitoes used was comprised of adults that had emerged from pupæ Nov. 12, 1915. They were fed as shown in Table II. After Nov. 20 they were fed on sugar alone. Nov. 21. Four mosquitoes were dissected. One contained five zygotes 19μ in diameter. Nov. 25. Five mosquitoes were dissected. Two contained zygotes; one contained one zygote, the other seven. Nov. 26. Dissected six mosquitoes. Two contained zygotes; one had one large zygote, the other seven or eight very large zygotes. One of these zygotes displayed sporoblasts; three of the other zygotes contained sporozoites. Nov. 28. Dissected four mosquitoes. One contained two large zygotes with sporozoites, another displayed three large zygotes with sporozoites.

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[6] Darling, S. T., Studies in relation to malaria, Panama, Canal Zone, Gatun. I. C. C. Press, 1916.

Thus out of nineteen mosquitoes that survived, seven, or 37 per cent, were positively infected. The jar still contained seven mosquitoes two or three of which were probably infected. The seven survivors were used in the inoculation experiments on Dec. 1, 3, and 4. On Dec. 6 the three that were left were killed and dissected. Two of the three contained a few sporozoites in the salivary glands and ducts.

Biting Experiment.—Dec. 1, 1915, 3 p.m. The jar containing infected *Anopheles ludlowi* was applied to Subject 1. Six of the seven mosquitoes fed. The feeding was interrupted and the same jar was now applied to Subject 2. Six mosquitoes were seen to feed. Judging from the degree of distension of their abdomens apparently they were the same ones that had just before fed on Subject 1. Dec. 3. The jar was applied to Subject 3, and three mosquitoes fed. Dec. 4. The jar was again applied to this patient and two mosquitoes fed.

TABLE II.

Time of feeding.	Patient No.	Gametes present in patient's blood.
1915		*per cent*
Nov. 16, a.m. and p.m.	4	3.9
" 17, p.m.	4	3.1
" 18, "	4	2.4
" 19, "	5	33.6
" 20, "	5	33.3

In none of the dissections of infected *Anopheles ludlowi* were zygotes ever encountered that showed signs of degeneration or delayed development. The species therefore offers a favorable medium for this plasmodium and is evidently an efficient host. As from 40 to 60 per cent of the mosquitoes were found to have become infected, we are safe in assuming that among the mosquitoes that bit the patients about half were infected and contained sporozoites. As a matter of fact, two of the three survivors at the conclusion of the experiment actually had sporozoites in their salivary glands. There is no certitude, however, as to the actual number of infected mosquitoes that bit the patients. Subjects 1 and 2 were certainly bitten by one and possibly by two or three infected mosquitoes. Subject 3 was bitten by one or possibly by two infected mosquitoes. Since two infected mosquitoes with sporozoites in their ducts were found after the conclusion of the biting experiments, it is likely that each man was bitten by at least two infected mosquitoes. They were certainly bitten by one, for each patient gave clinical evidence of infection.

Cases from Which the Mosquitoes Were Infected.

Dissections of mosquitoes in this experiment and others not recorded here indicate that the mosquitoes were infected from Subject 4 and not from No. 5. This belief is based on the failure to find small zygotes corresponding in size with those which should have been derived from No. 5. Every carrier of gametes is not infectious for mosquitoes. I pointed this out in 1908 and Dr. Barber and I have confirmed the fact several times since. In selecting a gamete carrier for incrimination experiments one with a sufficient number of gametes is necessary. From observations made in Panama in 1908 it would seem to me that at least twelve gametes per c.mm., or one for every 500 leucocytes, are necessary.

Infectivity of Anopheles ludlowi Infected under Experimental Conditions.

It can be asserted that when a gamete carrier has as few as 2.4 to 3.9 gametes per 100 leucocytes about 40 per cent of *Anopheles ludlowi* may be infected from him. These mosquitoes may have from one to eight zygotes and will furnish a sufficient number of sporozoites to infect man. The size of the salivary ducts offers no impediment to the free exit of sporozoites, and inoculation occurs with ease either in an interrupted feeding or after two previous feedings.

In view of these experiments and the positive epidemiological evidence advanced above *Anopheles ludlowi* must be regarded as one of the important carriers of malaria and must be included in the list of *Anopheles* against which antimalarial measures must be taken.

Power of Infected Mosquitoes That Have Already Bitten and Infected Man to Infect Others.

In an interrupted feeding mosquitoes first bit and inoculated Subject 1 and a few seconds later the same mosquitoes bit and infected Subject 2. 2 and 3 days later two to five of these mosquitoes fed on and inoculated Subject 3 with sporozoites without any demonstrable diminution of infecting material as measured by the symptoms. Moreover, surviving mosquitoes were found to contain sporozoites in their salivary glands.

We may assert that infected *Anopheles* may retain infection after at least three bitings. The biting habits and feeding exigencies of *Anopheles* are not thoroughly understood. That is, we do not know how many times an *Anopheles* may feed or how many times it is accustomed to feed between receipt and transference of infection. In the laboratory they may be induced to bite every other day. In nature they return to the breeding place after the blood meal has been digested. More or less lengthy flights are often necessary and feedings may occur only in response to demands for ovulation. The circumstances indicate that an *Anopheles* may remain infected throughout life and may infect at each feeding.

Period of Incubation.

The period of incubation as measured from the date of inoculation to the occurrence of fever was 14 days in Subject 1, and 15 days in Subject 3 if counted from December 3, the date of the first biting by three mosquitoes, or 14 days if counted from the date of the second biting, December 4. In Subject 2 the period of incubation was 18 days. This patient was already suffering from a tertian infection and the blood picture on December 12 and 13 indicated that the rises of temperature on December 13 and 14 were tertian paroxysms and not due to subtertian plasmodia. Subtertian malaria is not frankly exhibited until December 19 to 21, the access of fever coming on December 19 and many subtertian rings appearing in the blood on December 20. The period of incubation, therefore, was 18 days.

Rate of Appearance of Plasmodia in the Peripheral Blood and Its Correlation with Fever.

The rate of appearance of plasmodia in the peripheral blood will depend somewhat on the method of examination; thick films consisting of larger quantities of blood will disclose plasmodia earlier and in greater numbers than thin films. In Patient 3 on December 19 a thin film was negative, while the thick film was positive for plasmodia. A thick film represents about 1 c.mm. of blood. When plasmodia have become sufficiently numerous to be found in a film of this size they exist in large enough numbers to cause a paroxysm of fever. This was observed in all three cases.

Differences in the Clinical Picture and in the Spleen Reaction under Different Conditions.

Individual Infected for the First Time with Malaria.—Patient 1, infected for the first time, may have received a larger dose of sporozoites than the others. His blood, however, never disclosed an unusually large number of plasmodia, and his symptoms were rather disproportionately severe in relation to the number of plasmodia found. We know from abundant observations the truth of Manson's statements that the internal viscera are the organs involved in malaria and that the plasmodia in the peripheral blood often represent an overflow and are not an exact expression of the number of plasmodia actually present in the body. It may be asserted that the symptoms were more severe in this case of primary infection. The splenic enlargement began to manifest itself by tenderness on the 1st day of fever and at a time when plasmodia were first discovered in thick films. The spleen reached 1 fingerbreadth below the costal margin on December 19, or 4 days after the onset of fever. It returned almost to normal proportions December 24. In this case there is a correspondence in time between the appearance of splenic enlargement, the appearance of plasmodia in thick films, and the onset of fever, all of which occurred on or about the same day. The spleen remained enlarged to palpation only about 11 days.

Individual Infected after a Long Interval of Freedom from Malaria.—Patient 3 was the third case to be bitten and he may have received a smaller dose than the patient previously bitten. We note that the period of incubation was 15 or 14 days, or about the same as that of Case 1. The appearance of plasmodia in thick films on December 18 was accompanied by a rise in temperature, but the patient while showing evidences of infection was not really ill until December 25, or the 8th day of fever, whereas Patient 1 was acutely ill on the 5th day. No. 3 had a pronounced splenic reaction. The spleen which had been negative upon palpation was palpable just below the costal margin on December 17, or the day before the initial rise in temperature. It reached its maximum size 1 fingerbreadth below the umbilicus on December 26, the day that the temperature reached normal after the last rise in temperature, or 8 days after the initial rise in

temperature. Nearly the same degree of enlargement was present for several weeks later, for on January 18 it was still 1 inch above the umbilicus. Here we note a prompt reaction and prolonged period of enlargement, the degree of enlargement being that of splenomegaly and occurring in a man who had previously suffered from several attacks of malaria.

Individual Already Suffering from Tertian Malaria.—Patient 2 not only appeared to experience no inconvenience from the tertian infection from which he was suffering, but the additional infection of subtertian malaria likewise elicited no evidence of physical illness. The dose in this case can be assumed to have been at least as great as in Case 3 for he was the second man to be bitten. The period of incubation was 18 days. It is possible that the coexisting tertian infection influenced the duration of the period of incubation. Subtertian plasmodia appeared in large numbers on December 20 and in notable numbers before and after this date as well as could be ascertained. It is certain, therefore, that no immunity reaction had been provoked by the tertian infection which would tend to prevent the development of the superimposed subtertian infection. The spleen showed no response to either infection by tenderness or enlargement.

Type of Febrile Reaction and the Probable Invalidity of Plasmodium falciparum quotidianum as a Species Distinct from Plasmodium falciparum.

Text-figs. 1 to 3 not only display the type of fever seen during the period of incubation and access of fever, and the effect of quinine in reducing the fever, but they are of unusual interest in exhibiting the peculiar type of febrile reaction elicited in persons experimentally infected with a pure strain of subtertian plasmodium. The types of temperature graphs seen in clinical cases of malaria that have run for longer or shorter periods are often the result of multiple or superimposed infections. The phenomenon of acceleration or retardation of paroxysms often causes irregularly continuous curves. In malarial communities in the Tropics mixed infections are extremely common, and it is often difficult to diagnose the type of plasmodium by an inspection of the chart. Our knowledge of the types or species of ma-

larial plasmodia has been almost wholly advanced by studying the disease in persons who have been infected naturally, in whom the disease has run for a longer or shorter period of time, and in whom acceleration and multiple infection have complicated the clinical picture.

The study of the morphology of plasmodia is beset with difficulties inherent in a science which is limited to studies of form and staining reactions. There is usually little difficulty in differentiating most of the forms of plasmodia of quartan, tertian, and subtertian malaria. Young trophozoites and young gametes at times cause trouble. It is easy enough to follow the drawings of the text-books and assert because of resemblances of forms in the slide to pictured representation of what authors have chosen to call young tertian trophozoites or malignant tertian gametes that the former actually are identical with the latter, but whether they are absolutely so or not is an entirely different matter. For example, in the cases here described all the infections resulted from a case of malaria the blood of which contained no other forms but crescents. No tertian plasmodia were ever seen, yet when the experimental cases began to reveal plasmodia in their blood there were slight differences noticeable in some of the young trophozoites. When, for purposes of demonstrating the personal equation in the specific identification of plasmodia, these were shown to experienced microscopists, some trophozoites were called subtertian, others benign tertian. As none of these young "tertian" trophozoites ever matured into more adult forms, the assumption was that they were all subtertian as they should have been, and the microscopist was mistaken in his diagnosis as he undoubtedly is occasionally.

Text-books contain references to two kinds of *Plasmodium falciparum*; namely, a tertian and a quotidian variety. This is largely due to the accounts of two varieties of fever and plasmodia by Marchiafava and Bignami. Mannaberg, Manson, Grassi, Feletti, and Craig are of this opinion also. According to Marchiafava and Bignami, the quotidian variety causes a paroxysm every 24 hours. The fever may be regular like the benign tertian, but oftener exhibits acceleration or retardation, becoming irregularly remittent or continued.

The malignant tertian plasmodium of Marchiafava and Bignami resembles the quotidian very closely but according to them is larger and contains a greater amount of pigment. It may become one-half the size of an erythrocyte. The malignant tertian plasmodium possesses greater ameboid activity. The quotidian plasmodium is very pale; it never exceeds one-third the size of an erythrocyte. The infected erythrocyte has a brassy color, is often shrunken, and its hemoglobin is retracted.[7]

An inspection of the temperature charts of the two uncomplicated cases, Nos. 1 and 3, will show that before there has been any administration of quinine the initial paroxysms are tertian or subtertian in periodicity and that the subsequent ones are quotidian. Moreover, in each intermission of fever in the tertian phase there is a slight rise of temperature about 4 p.m., thus giving evidence of a latent quotidian tendency even in the tertian phase which soon manifests itself as a distinct quotidian wave which may be followed by a remission or definite intermission of fever. Thus we have the interesting phenomenon of a pure strain of subtertian plasmodia provoking in infected persons a febrile reaction which at first is distinctly tertian in periodicity. This is immediately succeeded by a reaction having quotidian periodicities.

If we accept the claim of Marchiafava and Bignami, and Craig that there exist two distinct forms of *Plasmodium falciparum*, the quotidian and the malignant tertian varieties, we may account for the phenomenon by assuming that the patient from which the mosquitoes were infected was suffering from both forms of the disease and that the gametes consisted in part of quotidiana and in part of tertiana maligna. But in that case it is difficult to understand why the fever did not manifest itself from the start in more pronounced quotidian form. This theory will not explain the well known tendencies of subtertian infection to exhibit irregularly remittent and continued forms of fever.

We may assume, on the other hand, that there is but one form and species of *Plasmodium falciparum* and that this species possesses inherently both subtertian and quotidian tendencies manifested in

[7] Craig, C. F., The malarial fevers, New York, 1909, 34.

types of fever at times having tertian and at times quotidian periodicities, and also possesses the well known tendencies to cause fever of an irregularly remittent or continued type. This assumption I think is in better agreement with the observed facts. We do not know the cause of the lack of uniform periodicity in *Plasmodium falciparum*, but it may be related to the following facts. Quartan plasmodia sporulate in the peripheral blood and have from 5 to 11 merozoites. In Panama the number was 5 or 6 and very uniform. In Malacca the number was near 11 and also uniform. Tertian plasmodia also show considerable uniformity in the number of merozoites possessed by segmenting plasmodia. In subtertian malaria, however, films from the spleen, marrow, and brain display a lack of uniformity in the number of merozoites, for they have been found to run from 10 or 12 to 42. This morphological heterogony may and probably does in some way correspond to the clinical polymorphism observed in malignant tertian malaria. From my experience in Panama and Malacca it has never seemed to me that there existed sufficiently cogent reasons for creating a specific plasmodium for clinical forms of quotidian æstivo-autumnal malaria.

The diagnosis of *Plasmodium falciparum quotidianum* was oftenest made during the period when fresh films only were examined and before the Romanowsky stains came into use, and the creation of a separate species probably rests on insecure and insufficient testimony. At any rate, the matter requires further elucidation by means of pure strains and inoculation experiments.

SUMMARY.

Three persons were experimentally inoculated with malaria by means of *Anopheles ludlowi* reared from larvæ and infected with a pure strain of subtertian plasmodium (*Plasmodium falciparum*), thus proving that there exists no mechanical impediment or obstacle to the free exit of sporozoites from the salivary ducts or proboscis.

In the dissection of infected mosquitoes there were no evidences of degenerated zygotes. Sporozoites appeared promptly in the salivary glands (9 to 12 days). Inoculation occurred with ease either in an interrupted feeding or after mosquitoes had been fed twice previously.

The period of incubation was 14 and 18 days. The clinical manifestations were more severe in the subject that had never been infected with malaria previously, while the splenic enlargement was most pronounced in the subject infected after a long interval of freedom from malaria. In a third subject already suffering from tertian malaria there was only the slightest evidence of physical illness elicited by the superimposed subtertian infection; his temperature, however, became duly elevated.

The type of febrile reaction in the two uncomplicated cases was at first tertian, becoming quotidian later, and this phenomenon in a pure strain leads strongly to the supposition that *Plasmodium falciparum* possesses inherently both tertian (or subtertian) and quotidian tendencies, as well as its well known tendencies to cause fever of the irregularly remittent or continued type.

The creation of a specific plasmodium to account for clinical forms of æstivo-autumnal or subtertian malaria having a quotidian periodicity is probably unwarranted.

In consideration of the facility with which this species can be infected and man inoculated experimentally, the occurrence of naturally infected wild specimens, and the positive epidemiological evidence, there should no longer exist in the minds of sanitarians any doubt as to its being a malarial carrier.

Operations against this species can therefore be recommended without reservation and should be carried out without delay.

I am indebted to Assistant Surgeon A. E. Durasamy and to my laboratory assistant Mr. Chong Yoon Chong for assistance in obtaining histories and recording clinical notes of the patients.

STUDIES ON BACILLUS MURISEPTICUS, OR THE ROTLAUF BACILLUS, ISOLATED FROM SWINE IN THE UNITED STATES.

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PLATE 24.

(Received for publication, May 21, 1920.)

No essential differences have been detected between the mouse septicemia bacillus described by Koch (1) and the rotlauf or swine erysipelas bacillus subsequently discovered by Loeffler (2). The studies to be reported here are on organisms obtained from swine which, if they had been isolated in Europe, would undoubtedly have been called rotlauf bacilli, but since swine erysipelas is a disease that has not thus far been recognized in the United States, and since Koch's organism was the first to be described, they are called mouse septicemia bacilli, or *Bacillus murisepticus*.

There are reports that organisms of this group have been isolated three times in this country. Smith (3) in 1885 obtained a culture from the spleen of a pig dead of hog-cholera, and Moore (4) in 1888 obtained a culture from the spleen of another pig affected with the same disease. Smith (5) in 1894 again obtained a culture from the cervical lymph node of a pig that presumably had hog-cholera. This last paper is of special interest, for in it we find the first description of scurvy in guinea pigs and a demonstration that such animals may be susceptible to organisms to which the normal guinea pig is immune. In addition, there is a discussion of the relation of feeding to disease, a subject about which even today we know very little, but the importance of which we are beginning to realize.

These organisms are not difficult to isolate, and if they were at all common in the bodies of pigs with hog-cholera they would have been reported more frequently. In Europe, and especially in Germany, there have appeared many reports concerning their presence in swine. Koch (1) obtained the mouse septicemia bacillus from mice that he had inoculated with putrid material. How many times he found it and how frequently it has subsequently been obtained in this way cannot be determined from the literature. In 1882 Loeffler (2) found similar organisms in the bodies of swine affected with rotlauf, rouget, or swine

erysipelas. They were present in great numbers not only in lesions of the skin but generally distributed in the body, and he regarded them as the cause of the disease. Pasteur and Thuillier (6) had previously studied rouget and described as the cause an organism which from their description is difficult to identify. That they cultivated the organism described by Loeffler is shown by the observations of Schütz (7) and Smith (8), both of whom found the mouse septicemia bacillus in the vaccine prepared by Pasteur for protecting against rouget. The organisms found by Loeffler resembled Koch's bacillus in their morphology, growth in gelatin, and in being virulent for pigeons, white and gray mice, but not for field mice. Olt (9) quotes Loeffler as finding that animals which had recovered from an infection produced by the injection of rotlauf bacilli were immune to *B. murisepticus* and *vice versa*. This power of one organism to immunize against the other has repeatedly been confirmed, and Rosenbach (10) and others have shown that rotlauf serum will protect against the mouse septicemia bacilli as well as against the organisms obtained from swine.

Rosenbach (10) and Preisz (11) found the mouse septicemia bacillus to be slightly larger and to grow more rapidly in gelatin than the organisms obtained from swine. Rickmann (12), on the other hand, studied over 100 cultures obtained from swine with rotlauf and found considerable variation in their morphology, so that he could not differentiate some of them from the one culture of mouse septicemia he had, which was the one used by Rosenbach. Stickdorn (13) found that the frequent transfer of a virulent rotlauf culture or its passage through a series of mice or pigeons changed not only the virulence but the character of the growth in gelatin, and Smith (5) found that the same cultural character was markedly influenced by the reaction of the medium.

When injected immediately after its isolation the rotlauf bacillus may be virulent for swine, but after it has been on culture media for any length of time or after it has been passed through some of the experimental animals it loses this virulence and then resembles *B. murisepticus* in that it fails to cause disease when injected into pigs.

Organisms that could not be differentiated from those found in swine with rotlauf have been isolated from chickens by Schipp (14) and from sheep with arthritis by Poels (15). In continental Europe bacilli of swine erysipelas have also been found in swine that were apparently normal. Bauermeister (16) inoculated fifteen mice with the secretion from the tonsils of fourteen pigs. From five of the mice he obtained cultures of what he called rotlauf bacilli. Olt (9) also found these organisms in normal swine, but he does not give the number of animals examined or the number of cases that were positive. Van Velzen (17) studied the tonsils of eleven normal pigs and from three obtained rotlauf bacilli. Pitt (18) inoculated mice with the secretion from the tonsils of 50 pigs and with the secretion from the glands at the ileocecal valve of 66 pigs. 56 per cent of these swine showed rotlauf bacilli in the tonsils and 40 per cent in the glands at the ileocecal valve.

That these organisms are the cause of rotlauf seems to be generally accepted. The fact that they are usually present in great numbers and the encouraging results obtained by vaccination are in favor of this view. It is, however, unusual to find such a large percentage of normal animals that are carriers of organisms that are the primary agent of a disease. Olt (9) has suggested that these bacteria may gain entrance to the body through lesions produced by intestinal parasites and then become virulent; Smith (5) emphasizes the relation of feeding to an invasion of the body. There is, furthermore, the possibility that the organisms found in normal swine differ from those found in rotlauf. Proof of any of these hypotheses is lacking and the facts as we now know them suggest that these organisms may be secondary invaders. There are diseases in which secondary invaders are almost invariably present and at times, due to their number or the ease with which they are isolated, are regarded as the primary agents of a disease. In some cases it is only by the most careful work and the use of special methods that the primary agent of the disease is recognized. The object of this paper is to call attention to the fact that these organisms occur in the United States where the disease of swine erysipelas is unknown, to give the cultural reactions of the organisms isolated, to record some observations on the pathology of the disease produced in experimental animals, especially the mouse, and finally to raise the question as to whether these bacilli really are the primary agent of rotlauf in swine.

Isolation of Cultures from Swine.

In a study of the tonsils of pigs that had been inoculated with hog-cholera virus some of the exudate from an ulcer on the tonsil was injected into mice. These mice died in a few days and from them were isolated organisms that corresponded to the mouse septicemia bacillus. Following this first observation, material from the tonsils of all the swine that came to autopsy was injected into mice, and when these animals died, cultures were made from the spleen and heart's blood and films from the lungs were stained by Gram's method and examined for the intracellular Gram-positive rods, which are characteristic of *Bacillus murisepticus*.

Sixteen swine, all infected with hog-cholera virus, have been examined in this way and from the tonsils of five of these bacilli have been isolated which morphologically and culturally are mouse septicemia bacilli. The organisms were apparently localized in the tonsils, for they were not found in cultures made by transferring pea-sized bits of liver, spleen, and kidney to agar slants.

Four of the positive swine were from the same litter and the source of the fifth one is questionable, so that it appears that the infection is restricted. On the farm from which these animals came there is no record of any diseases among pigs, and before inoculation the animals were apparently well.

These organisms may have some relation to the ulcers that are so commonly present on the tonsils of pigs with hog-cholera, as four of the five positive cases showed ulcers, whereas only four of eleven negative cases showed them. It is just as possible, however, that a variety of organisms may be associated with these ulcers, as they are probably due to invasion by bacteria of a lesion produced by the hog-cholera virus. Bacilli of the swine-plague group were often present and killed the inoculated mice before the mouse septicemia bacilli had time to invade the bodies of these animals. It would be of interest to know how common and how widespread the mouse septicemia bacilli are in the United States, and if such a study is made it would be well to immunize part of the mice used for their isolation by the injection of anti-swine-plague serum as was done by Van Velzen (17).

Cultural Characters.

The ease with which mouse septicemia bacilli are identified probably accounts for the meager description of their cultural reactions. The morphology has been well described, as has the growth on agar and in gelatin, but this is as far as most descriptions go. It therefore seemed worth while to gather together the recorded reactions and to fill in the most obvious gaps. The five cultures isolated recently and the one culture isolated by Smith (5) in 1894 have been used, and all have been found to give the same results.

Morphology.—These organisms are non-motile, Gram-positive rods, varying considerably in length and diameter, the variation depending

upon the source from which preparations are made. In films from mice, dead after inoculation with a pure culture, they appear as very slender, straight or slightly curved rods from 1.5 to 2 μ in length. They may appear free or are grouped in a characteristic manner in large cells with an indefinite nucleus. From the surface of Loeffler serum slants and in gelatin incubated at 37°C. they appear as straight or slightly curved rods somewhat thicker than those found in the tissues of mice. In bouillon, and especially on agar slants, this increase in size is more marked. The organisms may measure up to 4 μ in length and show a decided tendency to curve and form clumps of interlacing bacilli. Branching forms have not been found in films made from cultures on a variety of media incubated up to 5 days. In sections of tissues stained by the Gram-Weigert method the organisms often have a beaded appearance, but in films stained by Gram's method, using Stirling's gentian violet and decolorizing with alcohol, the organisms stain uniformly.

Agar.—Growth appears in 24 hours in the form of fine, translucent, slightly gray colonies. It is very scanty, but becomes more abundant if serum or defibrinated blood is added to the medium.

Gelatin.—Stab cultures show the characteristic "test-tube brush" growth described by Loeffler (19) which has been serviceable in identifying these organisms. Petri and Maassen (20) obtained the same type of growth in a semisolid medium at incubator temperatures, but I have been unable to confirm this. Smith (5) noted that when the gelatin was acid this characteristic growth did not occur. After some weeks incubation gelatin stabs show a finger-like depression which is not found in uninoculated tubes that are incubated the same length of time. If this is due to a softening and evaporation of the gelatin, as has been stated, it is evident that it proceeds at a very slow rate and that it only occurs in the presence of oxygen. Gelatin cultures that have been incubated at 37°C. for 30 days will still harden when placed in the refrigerator.

Bouillon.—In 24 hours there is a uniform turbidity and when shaken a characteristic cloud-like effect is produced that has been considered of diagnostic value.

Blood Serum.—Several writers state that these organisms do not grow on this medium, while Poels (15) states that it grows but does

not liquefy the serum, this being one of the differential points between the rotlauf bacillus and *Bacillus pyogenes*. All of my cultures have formed a scanty growth on Loeffler serum slants and after 30 days incubation there was no evidence of liquefaction.

Milk.—All writers agree that no visible change is produced in this medium. Moore (4) found microscopic evidence of vigorous growth of the one strain he studied, while Schipp (14) found no evidence of growth of the three strains with which he worked. All of my cultures have failed to produce acid in milk, yet all of them show microscopic evidence of growth. As will be noted below, these organisms attack lactose in bouillon, but not in milk.

Ammonia.—6 day cultures in fermented bouillon failed to show any appreciable amount of ammonia.

Hydrogen Sulfide.—Petri and Maassen (20) found this gas in their cultures, and in swine dying of rotlauf they found sulfmethemoglobin, but they did not find sulfmethemoglobin in experimental animals inoculated with pure cultures. All six of my cultures blackened lead acetate in peptone agar in 24 hours.

Indole.—Schipp (14) has the only reference that I have found to indole production, his three cultures all giving a negative test. All six of my strains in fermented bouillon cultures 5 days old gave a negative test with Ehrlich's aldehyde as did a typhoid culture and the uninoculated medium. A colon culture gave a positive reaction.

Phenol.—No reference has been found on phenol production. Three of my cultures were grown for 10 days, each in 40 cc. of fermented bouillon contained in 100 cc. Erlenmeyer flasks. They were then distilled with steam and the distillate was tested with bromine water and Millon's reagent. Reactions for phenol were not obtained.

Hemolysins.—Van Nederveen (21) found that his culture did not hemolyze swine blood agar in plates, or the blood of cattle, rabbits, or pigeons in bouillon. The six cultures of the present study all produced a distinct zone of hemolysis around the deeper colonies in veal infusion agar plates containing about 10 per cent of sterile defibrinated horse blood. The same blood in bouillon was not hemolyzed.

Fermentation of Carbohydrates.—The literature gives very little information about this important phase of the bacterial activity of these organisms, and that found is not in agreement. Fermi (22) states that

the rotlauf bacillus forms acid from starch but that it does not possess a diastatic enzyme. Petri and Maassen (20) found that the growth of their rotlauf cultures was increased when dextrose, lactose, saccharose, or dextrin was added to the medium. Smith (5) studied one culture and found that acid but no gas was formed in bouillon containing dextrose or lactose, while no acid was formed in bouillon containing saccharose. Schipp (14) studied three cultures and found that acid was formed in a litmus peptone solution containing lactose or saccharose, while in the same medium containing dextrose the litmus was decolorized but was not reddened.

Preliminary tests with fermentation tubes containing fermented bouillon plus 1 per cent of either dextrose, lactose, or saccharose

TABLE I.

Carbohydrate.	Initial reaction.		Length of incubation.	Final reaction.		
	Acidity.	pH		Acidity.		pH
	per cent		*days*	*per cent*		
Dextrose	0.7	7.5	5	2.7–3.1		6.1–5.8
Lactose	0.9	7.5	7	2.4–3.1		6.4–6.0
Arabinose	1.3	7.3	7	1.6–1.8		6.7–6.6
"	1.3		10	1.8–2.1		
" fermentation tubes	1.1		7	Bulb. 1.7–2.1	Branch. 1.1–1.5	

showed that these organisms did not form gas, but produced acid from the first two carbohydrates and that somewhat more acid was produced in the presence of oxygen than in its absence. In saccharose bouillon there was no acid or alkali formed. The great majority of the tests has been made in ordinary test-tubes containing 13.5 cc. of fermented bouillon to which 1.5 cc. of a previously autoclaved 10 per cent solution of the carbohydrate in distilled water was added. The tubes were steamed and incubated to insure sterility and were then inoculated from young bouillon cultures and titrated after from 5 to 7 days incubation. When acid was produced hydrogen ion determinations were also made, but when the carbohydrate was not acted upon this was not done. In Table I are given the extremes of reaction produced by the six cultures when acid was produced, the actual amount

of acid formed being the difference between the initial and final reactions of the medium.

It is evident that dextrose and lactose are attacked. The small amount of acid formed in the arabinose medium might indicate that there were impurities present which were acted upon while the carbohydrate itself was not attacked. The lot of arabinose used was the best obtainable and had been used for some time with satisfactory results in differentiating the hog-cholera bacillus from the other paratyphoids. If the mouse septicemia bacilli attack it at all they do so very slowly.

Fermented bouillon cultures containing the following carbohydrates have also been titrated and no acid has been found.

Xylose.	Inulin.
Dulcitol.	Salicin.
Maltose.	Dextrin.
Mannitol.	Starch.
Saccharose.	Glycerol.

Reduction.—The one attempt to study the reducing powers of these organisms was a failure as the presence of 1 per cent of either rosalic acid or methyl red apparently inhibited growth.

Pathogenicity.

Considerable work has been done on the virulence of the mouse septicemia bacilli for a variety of animals. Koch (1) found that they produced a fatal disease in white and gray mice, while the field mouse was immune. Loeffler (2) found that pigeons and sparrows were as susceptible as mice, and that frogs, salamanders, chickens, dogs, cats, and white rats were immune. In rabbits an erysipelas-like infection of the ears and a loss in weight followed an intravenous injection of the bacilli, but the animals usually recovered and later were immune. The virulence of the organisms isolated from pigs with rotlauf corresponds to that of the mouse septicemia bacillus except that very freshly isolated cultures may infect swine. This virulence is soon lost and the cultures are then the same as the organism isolated by Koch.

The strains which I have isolated are all virulent for white mice in as small amounts as 0.001 cc. of a 24 hour bouillon culture. The mice

are usually dead by the 3rd day. The culture isolated by Smith 26 years ago is also virulent for mice in the same amounts, but the animals die from 2 to 3 days later. These cultures when injected subcutaneously into pigeons cause death in about 4 days. Intravenous injection into rabbits causes a marked edema of the ear on the side of the injection and at times the opposite ear is also involved. The animals are very quiet, show a rise in temperature to around 41°C., and lose from 150 to 250 gm. in weight. Only one of the cultures will kill rabbits when injected either intravenously or subcutaneously. Whereas in mice and pigeons after death the organisms are abundant in all the organs and in the blood stream, in rabbits they are very scarce. They are rarely found in films, and in cultures made from bits of the various organs, or from several drops of blood, growth in agar slants occurs only in the condensation water.

Three pigs have been inoculated with the cultures isolated from the tonsils. One was given an intravenous injection of 1 cc. of a 24 hour bouillon culture. There was a slight rise in temperature without any signs of illness. The skin was normal and the appetite undiminished. The pig was then inoculated with hog-cholera virus and at autopsy cultures were made from the liver, spleen, and kidney, with negative results.

Two pigs were inoculated with another strain, one receiving 5 cc. of a 24 hour bouillon culture intramuscularly and the other 4 cc. intraperitoneally. Neither pig showed any effects from the inoculation. These results correspond to those obtained by Smith (5) and Moore (4) with the mouse septicemia bacilli which they isolated in this country.

One of the striking features of films made from mice and pigeons is the great number of organisms that is found in leucocytes. Koch (1) first called attention to this, and it has since been used as one of the means of identifying these organisms. Curiously enough, I have found no statement as to the type of cells in which these bacteria are found. From the drawings of Koch (1) and Moore (4) one might assume that they are polymorphonuclear leucocytes, since some of the cells show two nuclei. In the literature they are called leucocytes without further qualification.

Several years ago I made some experiments with an old stock culture of the mouse septicemia bacillus, one of the objects being to determine in which type of cell it occurred. After a subcutaneous injection of about 0.01 cc. of a 24 hour bouillon culture mice died on the 4th or 5th day. A number of inoculated mice were killed at intervals, and films, sections, and cultures made from the various organs. Cultures showed that the organisms were generally distributed at the end of 2½ days, but they were so scarce that they could not be found in films. On the 4th day, when the animals appeared to be sick, the bacilli could be found in films, especially in those from the lungs. Except when present in great numbers, they were always intracellular, and the only type of cell in which they could be found was the endothelial leucocyte (Figs. 1 and 2). Sections of the various organs showed that the bacilli at this stage were in the endothelial cells lining the veins and capillaries and also in endothelial cells free in the blood stream (Figs. 4 and 5). They were not found in endothelial cells lining the arteries or the heart. As the time of death approached the organisms were present in enormous numbers, filling the endothelial cells and crowding the nucleus to one side so that the cell resembled a sac containing a culture of the bacilli. When films were made these sacs were ruptured and the organisms were set free.

In pigeons the process was apparently the same except that the organisms were found generally distributed about 24 hours earlier than in the mouse. They were in the endothelial cells lining the veins and capillaries, and the type of cell in the blood containing them was apparently an endothelial leucocyte (Fig. 3). In both the mouse and the pigeon the organisms were at no time found in polymorphonuclear leucocytes.

Endothelial cells with only a few bacteria soon show evidences of injury. The cytoplasm contains small vacuoles and the nucleus when stained with a modified Romanowsky stain has a more reddish tinge than the nucleus of the same type of cell that is free from bacteria. As the bacilli become more numerous larger vacuoles appear in the cytoplasm and the nucleus stains distinctly red and shows evidences of disintegration.

The disease in the mouse appears, then, to be associated with an intracellular process. The organisms are taken up and instead of

being destroyed are able to multiply and finally to kill the cell. Some of these infected endothelial cells probably break away from the walls of the vessels and float free in the blood stream. Rous and Jones (23) have called attention to the fact that phagocytosed bacteria may be protected against immune substances in the body fluids. There are other instances of the multiplication of bacteria in cells. The leprosy bacillus may be found in the endothelial cells of blood vessels and the tubercle bacillus and *Treponema pallidum* are usually intracellular. Smith (24) has called attention to the localization of *Bacillus abortus* in the chorionic epithelial cells of cattle, and Tyzzer (25) to a disease of Japanese waltzing mice in which the organisms are found in the liver cells and cells of the intestinal mucosa. It is worthy of note that most of the diseases in which the organisms are found in cells are more or less slowly progressive, while the disease in the mouse produced by *Bacillus murisepticus* is acute.

SUMMARY AND CONCLUSIONS.

In the United States organisms, which culturally are mouse septicemia or swine erysipelas bacilli, have been isolated from the tonsils of five of sixteen pigs examined. These pigs all had hog-cholera, but it is probable that the bacilli were in the tonsils before they were infected with hog-cholera, and there is no evidence that they played any part in the disease. The distribution of the infection seemed to be restricted as most of the pigs from which the bacilli were obtained came from one litter. As we do not have clinical rotlauf, or swine erysipelas, in this country, as these organisms, in Europe, have been found in a large percentage of apparently normal swine, and as the disease is produced with difficulty by the injection of cultures, the question may be raised whether they are not secondary invaders rather than the primary cause of the disease with which they have been associated, or else whether the resistance of swine on the European continent does not differ from that of our breeds as a result of differences in foods.

It is possible that the mouse septicemia bacilli found in this country may differ culturally from those present in animals with swine erysipelas. With this in mind, the carbohydrate reactions, as well as

other cultural characters not necessary for the identification of the bacilli isolated, have been studied.

The disease produced by the injection of these bacilli into mice and pigeons has been studied and shown to be largely an intracellular process. The organisms are taken up by the endothelial cells lining the veins and capillaries; there they multiply and soon kill the cells. It has also been shown that the only type of cell in the blood stream which contains bacteria is the endothelial leucocyte, and the probabilities are that the free phagocytes have been detached from the lining of the vessels. The disease is acute, and the indications are that in the cells the bacilli find a favorable medium for their growth. While phagocytosis may in general be an immune reaction, in this case it appears to favor the parasite rather than the host.

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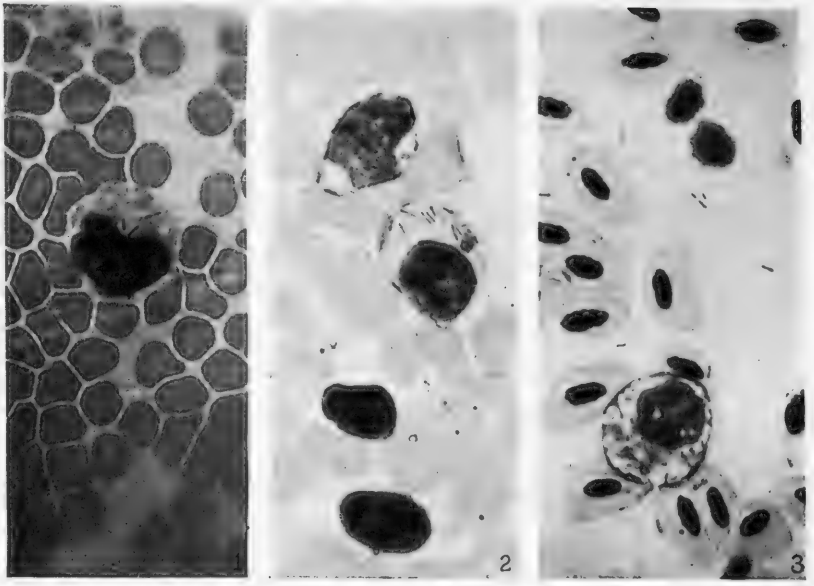
EXPLANATION OF PLATE 24.

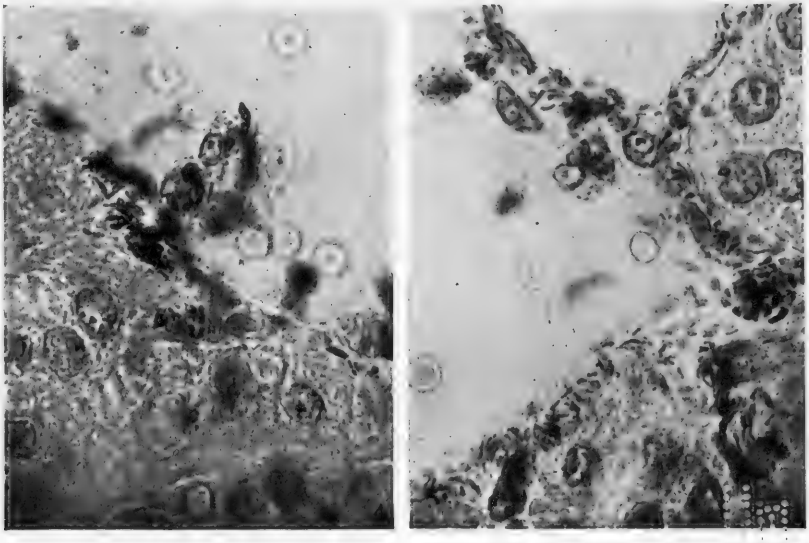
FIG. 1. Film from the heart's blood of a mouse killed 3 days after the subcutaneous injection of 0.01 cc. of a bouillon culture of freshly isolated *B. murisepticus*. Bacilli in an endothelial cell. × 1,000.

FIG. 2. Film from the lung of the same mouse. Bacilli in two endothelial cells. × 1,000.

FIG. 3. Film from the lung of a pigeon dead after a subcutaneous injection of 0.01 cc. of a bouillon culture of freshly isolated *B. murisepticus*. Bacilli in a mononuclear cell which is probably an endothelial leucocyte. Free organisms from ruptured cells. × 1,000.

FIGS. 4 and 5. Sections of the kidney of a mouse showing *B. murisepticus* in the endothelial cells of a vein. × 1,000.





(TenBroeck: *Bacillus murisepticus.*)

EFFECTS OF ENZYMES IN SERUM ON CARBOHYDRATES AND THEIR RELATION TO BACTERIOLOGICAL TECHNIQUE.

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While studying the carbohydrate reactions of the mouse septicemia bacillus (1) a source of error was detected which apparently has not been appreciated by some bacteriologists and to which it is the object of this paper to call attention. These organisms grow better when a small amount of serum is added to media. Therefore, according to the custom of those who have been working with streptococci, a few drops of sterile horse serum were added to each tube of carbohydrate bouillon at the time the inoculation was made. Under these conditions acid was produced from dextrin and starch, whereas some of the simpler carbohydrates were not attacked. The possibility that this action might be due to enzymes in the serum which made the carbohydrates available to the bacteria was considered and the following experiments were made.

That various sera contain carbohydrate-splitting enzymes has been known to physiological chemists for years and the fact is stated in a number of text-books. Magendie (2) in 1846 found a starch-splitting enzyme in serum and Hamburger (3) showed that in addition to a diastase, a maltase was present. Moeckel and Rost (4) studied the sera of most of the domestic animals and found that they all contained a diastase. Invertase may be present in small amounts in some sera, though Abderhalden and Rathsmann (5) did not find it in dog serum until after they had injected saccharose into the circulation.

Among bacteriologists, Hiss specifically states that serum water should be heated to destroy the enzymes before the carbohydrate is added. Holman (6) found that streptococci grow better when unheated serum is used, and he introduced a medium containing 20 per cent of serum that is sterilized by filtration. Park and Williams (7) add an equal amount of sterile serum or ascitic fluid to a previously sterilized peptone water containing the carbohydrate to be used.

While the use of serum waters for the detection of acid is being gradually replaced by more accurate quantitative methods, the practice of adding a few drops of serum as an enriching substance is not at all uncommon. That this practice may give rise to misinterpretations is indicated by the following experiment.

Experiment 1.—Four fermentation tubes were prepared, one containing sugar-free bouillon, and the other three the same bouillon plus 1 per cent of Kahlbaum's soluble starch. Horse serum was added as indicated in Table I and the tubes were incubated over night to insure sterility. They were then inoculated from a bouillon culture of Morgan's Bacillus No. 1, an organism that attacks only the hexoses. After 5 days incubation the amount of gas was recorded and the amount of titratable acid was determined in the fluid of the bulb and of the branch of each

TABLE I.

Effect of Horse Serum on Starch.

Fermentation tube containing sugar-free bouillon plus.		Results of inoculation with Morgan's Bacillus No. 1 and incubation for 5 days.		
			Reaction of.	
Starch.	Serum.	Gas.	Bulb.	Branch.
per cent		*per cent*		
1	None.	None.	Neutral.	Acid, 1.3 per cent.
1	1 cc. = 4 per cent.	30	Acid, 2.3 per cent.	" 3.5 " "
1	2 " = 8 " "	23	" 3.7 " "	" 3.3 " "
None.	2 " = 8 " "	Bubble.	" 0.5 " "	" 1.3 " "

tube. The results obtained are given in Table I and show that in the presence of serum or starch alone gas and acid are not formed, whereas when both of these substances are present both gas and acid are produced.

Since in the above experiment the smallest amount of serum used was approximately 4 per cent, another experiment was made to determine the least amount of serum that would act on starch.

Experiment 2.—To tubes containing 4 cc. of sterile 1 per cent soluble starch was added 1 cc. of various dilutions of sterile horse serum. After 2 days incubation 0.5 cc. from each tube was transferred to a tube containing 5 cc. of Benedict's solution and the tube heated to boiling in a water bath. Tests for bacterial growth in the original tubes were also made. The results of this experiment, given in Table II, show that 1.2 per cent of serum will attack the starch to such a de-

gree that in 2 days there are substances present that will give marked reduction of Benedict's solution, and with as little as 0.3 per cent serum there is still evidence of an hydrolysis of the starch.

That these enzymes are not affected by keeping sera in the refrigerator for a considerable length of time is shown by the fact that the serum of a horse, a cow, and a pig, each $1\frac{1}{2}$ years old, when added to starch solution caused, after 2 days incubation, the production of substances that reduced Benedict's solution. Heating horse serum

TABLE II.

Amount of Serum Required to Produce Reducing Substances for Starch.

Tube.	4 cc. of 1 per cent starch plus 1 cc. of horse serum.			Result of transferring 0.5 cc. to 5 cc. of Benedict's solution and boiling.	Test for bacterial growth.
	Dilution.	Serum.			
		per cent			
A	Undiluted.	20		Marked reduction.	Negative.
B	1:2	10		" "	"
C	1:4	5		" "	"
D	1:8	2.5		Reduction.	"
E	1:16	1.2	Incubated	"	"
F	1:32	0.6	2 days.	Slight reduction.	"
G	1:64	0.3		" "	"
H	1 cc. of distilled water (control).			No reduction.	"
I	1 cc. of serum plus 4 cc. of distilled water (control).			" "	"

to 50°C. for 15 minutes did not destroy the enzymes, but serum heated to 53° for 1 hour or 60° for 15 minutes no longer formed reducing substances in starch solution.

The effect of serum on carbohydrates other than starch was tested bacteriologically as follows:

Experiment 3.—Tubes, each containing 9 cc. of fermented bouillon, were autoclaved, and to sets of four was added 1 cc. of previously autoclaved aqueous 10 per cent solution of a carbohydrate to a tube. Eleven of the more commonly used carbohydrates were available for the study. It was realized that some of them, such as the pentoses and alcohols, would probably not be changed by the serum. To each of two tubes of a set was added 0.5 cc. of sterile horse serum, and all the tubes

were incubated for 3 days. Two tubes from each set, one with and one without serum, were then inoculated from a bouillon culture of Morgan's Bacillus No. 1 and the other tubes from a culture of *B. dysenteriæ* Shiga; both of these attack only the hexoses. After a further incubation of 2 days the contents of each tube were titrated and the results given in Table III were obtained.

These results show that serum will change maltose and dextrin so that they give the same bacteriological reactions as dextrose. There is a slight breaking down of saccharose, but other tests have shown that it is of little importance, and while it is possible that other carbohydrates may be changed though not broken down

TABLE III.

Effect of Horse Serum on Various Carbohydrates.

Fermented bouillon plus 1 per cent.	Titratable acid after inoculation with.			
	Morgan's Bacillus No. 1.		*B. dysenteriæ* Shiga.	
	5 per cent serum.	No serum.	5 per cent serum.	No serum.
	per cent	*per cent*	*per cent*	*per cent*
Arabinose	1.0	0.8	1.1	1.1
Xylose	1.0	1.0	0.9	No growth.
Mannitol	0.7	0.5	1.0	0.9
Dulcitol	0.7	0.6	1.1	1.0
Lactose	0.8	0.8	1.0	0.9
Saccharose	1.7	0.7	1.3	1.0
Maltose	4.4	0.5	3.8	1.0
Raffinose	0.8	0.6	0.9	0.7
Inulin	1.0	0.5	1.3	1.2
Dextrin	3.0	0.8	3.9	0.9
Salicin	0.8	0.6	1.1	1.1
Distilled water	0.8	0.6	0.9	0.8

to a hexose, we have no evidence that this takes place. It is fortunate that inulin and salicin are not attacked, as these two carbohydrates have been used so much in differentiating streptococci.

Maltose, dextrin, and starch are, then, in the presence of unheated serum, of no value in differentiating bacteria. They may, however, be of great value when used without serum. Maltose has not been generally used, since it often gives the same results as dextrose. In separating the fowl typhoid from *Bacillus pullorum*, maltose is of great value and it also helps in classifying the dysentery bacilli.

CONCLUSIONS.

It has been shown that enzymes in serum will change maltose, dextrin, and starch so that they will react as dextrose in media. These enzymes are destroyed by heating to 60°C. for 15 minutes, but they are present in sera that have been refrigerated for as long as 18 months. The practice of using carbohydrate media containing unheated serum should be discouraged, and if it is used the possibility that the carbohydrate may be changed by the enzymes present must be considered.

The writer is indebted to Mr. Henry Hagens, of this laboratory, for valuable technical assistance.

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A MODIFICATION OF VAN LEERSUM'S BLOODLESS METHOD FOR RECORDING BLOOD PRESSURES IN ANIMALS.

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Plates 25 to 28.

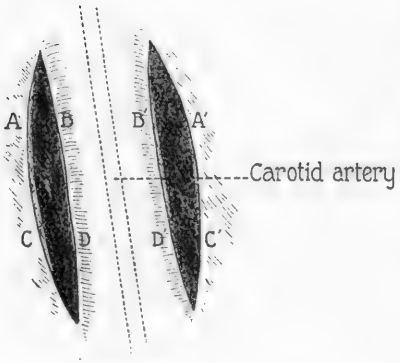
(Received for publication, May 17, 1920.)

A satisfactory method does not exist for taking the blood pressure in laboratory animals so that observations can be repeated on successive days over long periods of time. The advantages of possessing such a method are obvious. A single animal may behave differently on different occasions; it may react differently to different members of the same group of agents. A method such as the one described in this communication affords an opportunity for ascertaining the usual behavior of an animal.

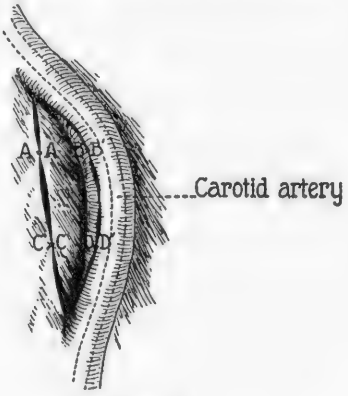
The essential part of the method was described in 1911 by Van Leersum[1] and was utilized by him for studies on rabbits. It consists in making the carotid artery accessible to direct examination. In order to do this an aseptic operation under ether anesthesia is performed. Two longitudinal and parallel incisions are made in the neck of the animals (dogs were utilized by us) about 7 to 10 cm. long (Text-fig. 1), the distance between the two being about 3 to 4 cm. The stretch of skin between the two incisions is freed from the underlying muscle. As much of the subcutaneous tissues is permitted to remain attached to the skin as will insure to it a sufficient blood supply. The carotid artery is then found and gently freed of the other structures contained in the carotid sheath; when possible a generous amount of areolar tissue is permitted to continue to surround the artery. The length of the artery freed in this manner should be

[1] Van Leersum, E. C., Eine Methode zur Erleichterung der Blutdruckmessung bei Tieren, *Arch. ges. Physiol.*, 1911, cxlii, 377.

several centimeters longer than the skin incisions. The edges (Text-fig. 2) of the stretch of skin are next sewed together so that the skin surrounds the artery as a tube. The outer edges of the two incisions are then approximated and sutured to restore the skin of the neck. The adjustment of the edges at the two ends of the tube and of the skin of the neck must be exact. The ligature material may be silk, but we have found retaining sutures of chromic gut and intervening



TEXT-FIG. 1.



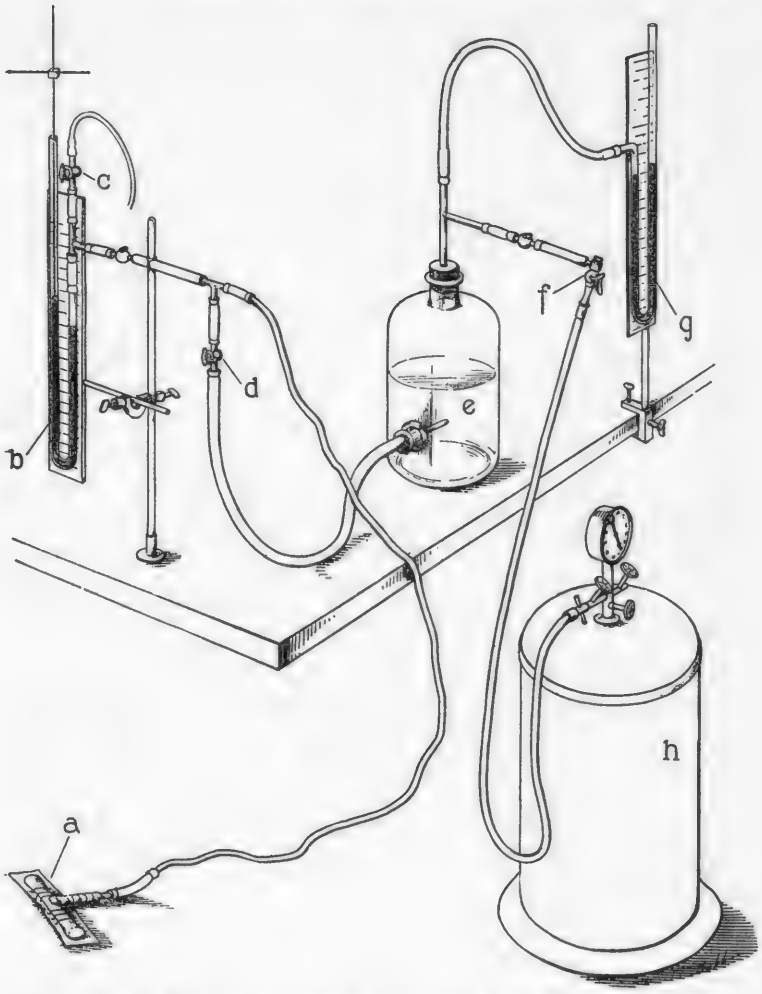
TEXT-FIG. 2.

TEXT-FIG. 1. Two vertical and parallel incisions in the neck are shown. The position of the carotid artery in respect to the stretch of skin between them is indicated.

TEXT-FIG. 2. The formation of the tube of skin containing the carotid artery is shown. After freeing the artery from the carotid sheath it is lifted out and surrounded by the stretch of skin between the incisions shown in Text-fig. 1. The operation is completed by suturing the points *BB'*, *DD'* and *AA'*, *CC'*.

sutures of plain gut more satisfactory. A dry dressing is interposed between the carotid tube and the repaired neck. The wound is dressed with dry gauze and the bandage is held in place with adhesive plaster. The wound may be dressed after an interval of 4 to 6 days. The sutures are removed when sufficient healing has taken place. The wound should heal by primary intention (Fig. 1).

The carotid artery surrounded by its tube of skin is now accessible to examination. In the technique employed by Van Leersum it is



TEXT-FIG. 3. Diagram of the cuff system. *a*, the cuff for surrounding the carotid tube. *b*, mercury manometer and writing point. *c*, valve permitting escape of pressure from the system. *d*, valve for elevating pressure within the system. *e*, water bottle kept at a pressure of about 250 mm. of mercury (*g*). *f*, escape valve. *h*, air pressure tank to supply pressure for *e*.

surrounded by a rubber cuff designed like that of von Recklinghausen but of smaller dimensions, 1 or 2 cm. wide by 3 or 4 cm. long. The pressure in the cuff is elevated until pulsation is no longer felt in the artery distal to the cuff. This pressure is read on a mercury manometer.

We have introduced a graphic method of recording pressure. The system between cuff and manometer is filled with water (Text-fig. 3). Except where it was impossible, we have used lead tubing. Pressure is introduced through a cock (*d*) into the system from a bottle kept at a pressure of about 250 mm. of mercury. It is allowed to escape through a stop-cock (*c*) at the proximal limb of the manometer.

By means of these cocks pressure can be elevated or can be permitted to fall either stepwise (Fig. 2) or gradually (Figs. 3 and 4). The record is made on smoked paper by a writing point supported on a float on the mercury column. The base-line is inscribed. Minimum and maximum oscillations utilized by Erlanger as the points of maximum and minimum pressures may be utilized.[2] We have obtained deflections of sufficient size to permit us to make accurate observations. If it is desirable, it is possible to train dogs to lie quite still for an hour or more without anesthesia or narcotics. To be able to do this has obvious advantages.

We are now making use of the method for the study of drugs of the digitalis series in which we desire to obtain simultaneous blood pressure records and electrocardiograms. The results of these studies will be given in another communication.

[2] To avoid the oscillations due to the shock of the impinging blood stream on the cuff, a second cuff distal to the first may be introduced about the carotid tube. The pressure of this cuff is maintained at the diastolic level. Only when the pressure in the artery is high enough to pass the proximal cuff, will the distal one be affected and a record be inscribed.

EXPLANATION OF PLATES.

PLATE 25.

FIG. 1. The carotid tube of one of the dogs after recovery.

PLATE 26.

FIG. 2. Below is the base-line. The blood pressure is inscribed by the method of intermittent escape of pressure. The systolic pressure is recorded at the step below the top (202 mm. of mercury); diastolic pressure at 100 mm.

PLATE 27.

FIG. 3. Record of blood pressure taken by the method of gradual escape. The systolic pressure is 154 mm. of mercury; the diastolic pressure is 88 mm. The top line is a signal to indicate when an electrocardiogram was made. The bottom is the base-line.

PLATE 28.

FIG. 4. Record of blood pressure taken by the method of gradual increase of pressure. The systolic pressure is 160 mm. of mercury; the diastolic pressure is 88 mm. The top line is a signal to indicate when an electrocardiogram was made. The bottom is the base-line.

FIG. 1.

(Cohn and Levy: Blood pressure in animals.)

FIG. 2.

(Cohn and Levy: Blood pressure in animals.)

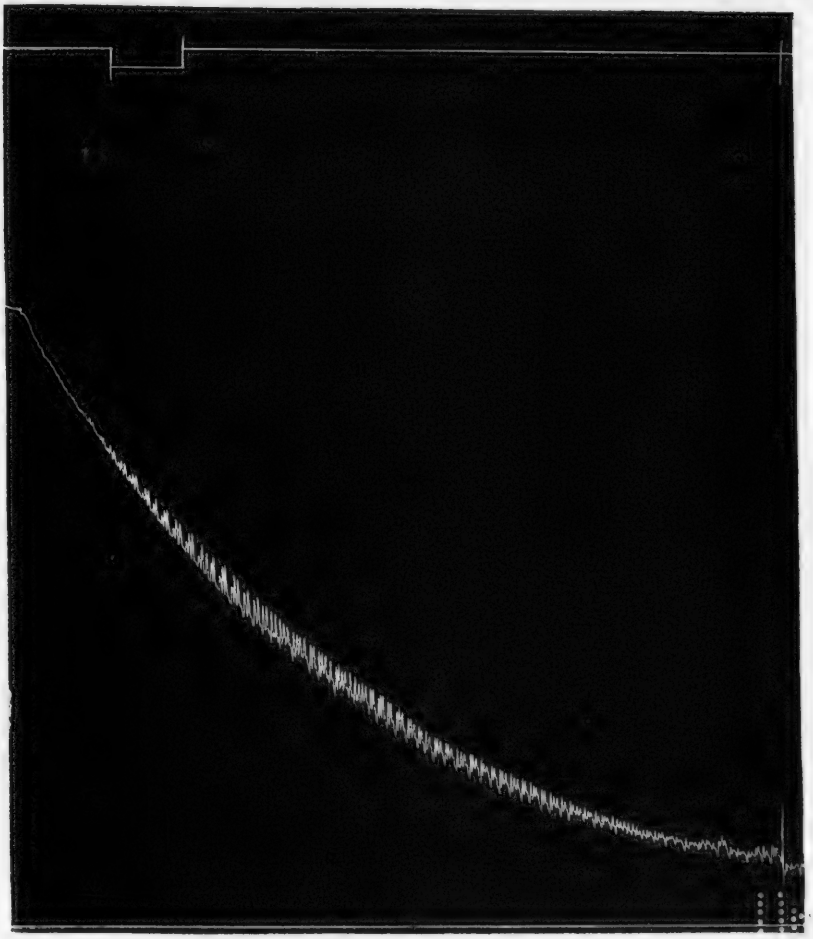


FIG. 3.

FIG. 4.

REPRODUCTION IN VITRO OF ENTAMŒBA TETRAGENA AND ENTAMŒBA COLI FROM THEIR CYSTS.

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PLATES 29 TO 34.

(Received for publication, December 16, 1919.)

The attempt to cultivate parasitic amebæ on artificial media by employing bacteriological methods, as in the case of non-parasitic amebæ, has produced a voluminous literature during the past few decades. The first cultivation in a fluid medium is claimed by Cunningham (1881). Later Maurice and Walker (1913) attempted to obtain a pure culture of amebæ from a liver abscess and from the intestinal contents of a typhoid patient by the addition of autolyzed liver and kidney to agar plates; but their attempts proved unsuccessful. Williams and Calkins (1913) cultivated amebæ on a rich brain medium; they added daily to this medium a quantity of blood in the presence of hemoglobinophilic bacteria, at a temperature of 38°C. The success of these investigators was due to the fact that they employed living bacteria in the cultivation of amebæ. It is possible that some other types of amebæ have been cultivated from the feces of patients, but whether or not these amebæ were unquestionably parasitic in character is doubtful. Kruse and Pasquale (1894), after numerous trials, concluded that the free living amebæ are capable of cultivation, while the parasitic amebæ are not, but opinion has been divided on this point.

For the past 2 years the writer has endeavored to obtain pure cultures of dysenteric amebæ. Professor Inada suggested that while it is impossible to cultivate successfully the vegetative forms of amebæ because they succumb easily as the bacteria in the medium grow, good results might be achieved with the use of cysts, which are far more resistant than the vegetative forms. On the basis of this suggestion experiments were made at room temperature with cysts relatively free from bacteria. The cysts were placed in a cat liver infusion and cat liver tissue medium according to Carrel's technique, and incubated at 37°C. Unfortunately, because of putrefaction of the culture medium, the experiments were unsuccessful. Since then an attempt has

been made to demonstrate *in vitro* the manner of development within the cyst of the vegetative form. In October, 1917, it was possible to show the reproduction process of the cysts of *Entamœba tetragena* as well as *Entamœba coli*.

On the basis of the experimental results, it may be stated that the cultivation of parasitic amebæ in the human intestinal tract is not at all improbable. The reproduction of these two amebæ takes place by a new process of fertilization; *i.e.*, a kind of autogamy. Moreover, it is evident in the case of these two amebæ that schizogony does not occur, as assumed by Casagrandi and Barbagallo, Schaudinn, and other investigators, for only a single vegetative individual arises from a cyst. Many protozoa pass through a process of sexual fertilization in their developmental cycle, but autogamy has not been observed heretofore with amebæ.

EXPERIMENTAL.

For the successful cultivation of the amebæ from their cysts, it was necessary first to ascertain the resistance of the cysts to certain chemical substances. Kuenen and Schwellengrebel (1913) claim that eosin is a delicate reagent for determining whether or not cysts are alive. Living cysts, they believe, do not stain when placed in a diluted eosin solution, while dead cysts will absorb the eosin instantly. By the aid of this method we were able to prove that bichloride of mercury in a concentration of 1:1,000 kills all the cysts after 4 hours treatment. 50 per cent alcohol and boiling water, and also Schaudinn's sublimate alcohol, kill them instantly. On the other hand, 10 per cent formaldehyde, acting for only a few minutes, does not kill the cysts.

These experiments show, however, that the eosin method does not absolutely establish the death of the cysts. While morphologically the cysts are shown to be dead following the effect of a mercuric chloride, alcohol, or formaldehyde solution, they frequently fail to take the eosin stain. The cysts were kept for 3 minutes in a sublimate solution of 1:1,000 after removal from a tissue or plasma culture medium at room temperature, according to the Carrel method. They were removed from this medium daily and stained with eosin and iron-hematoxylin in accordance with Heidenhain's method. On the 4th or 5th day it was observed that the majority of the cysts did not take

the eosin stain, although their nuclear and plasmic structures in the stained preparations were shown to have degenerated or to have disappeared. From this we may conclude that cysts taking the eosin stain are dead, but not, on the other hand, that all dead cysts absorb this stain.

Uzihara believes that it is possible to determine the resistance of the cysts to chemical agents by a vital stain of diluted neutral red solution, but we were unable to confirm his results. In accordance with Adachi's results, we observed that dead cysts stain slightly with the neutral red, while this is not the case with the living cysts, and that the vegetative forms take only the vital stain. As already stated, we still lack a simple method for determining the resistance of the cysts, and are obliged to obtain this information from animal experiments or the morphological properties revealed by the preparations.

The method followed is described below. A number of cysts were immersed for 5 minutes in a 2 per cent formaldehyde solution, while another set of cysts was immersed for the same length of time in a 2 per cent hydrochloric acid solution. After one washing, the cysts were placed in an isotonic salt solution. The two sets of cysts, together with the control cysts which from the beginning were placed in isotonic salt solution, were kept at room temperature. At intervals of 24 hours a small portion was removed and observed in a preparation stained with eosin or with iron-hematoxylin. In this way it was possible to determine that the majority of the cysts treated with hydrochloric acid, like the control cysts, remained morphologically unchanged; *i.e.*, that the majority of the cysts was not killed by being treated for 5 minutes with a 2 per cent solution of hydrochloric acid.

The material for these investigations was taken from the formed stools of a patient convalescing from amebic dysentery and two carriers of *Entamœba coli*. The feces appeared to be of normal consistency. Numerous cysts were always present in the discharges, but vegetative or other forms of amebæ were absent. It was proved previously that with the use of a special method encystment of the dysenteric amebæ may take place *in vitro* (Yoshida, 1918, *a*). A procedure almost identical with that formerly employed was used also in this case, but the results were negative. With the use of the

same method at a temperature of 28–30°C., we were able to observe *in vitro* the emergence of *coli* amebæ from their cysts; but the results were so meager that we were unable to show the reproductive process in the fixed preparations. Hence it was necessary to find another method, which is described below.

A portion of feces about the size of a thumb joint is placed in a beaker and covered with distilled water, sterile isotonic salt solution, or Ringer's solution, so that it forms a homogeneous mixture of about 40 cc. The water from this mixture is filtered through gauze and the filtrate is distributed in test-tubes and centrifuged by a hand centrifuge.[1] This procedure is repeated several times until the water is quite clear. In order to avoid injuring the cysts in mixing, a pipette with a rubber bulb was employed to admit air and thus distribute the portion of feces evenly.

The cysts are extracted from the sediment by a method based on the difference in specific gravity of the cysts and Mizuame's (Japanese) solution. The procedure is as follows: The sediment is placed in sterile Mizuame's solution, the specific gravity of which is 1,080, then mixed, and centrifuged. As the specific gravity of the cysts of *Entamœba tetragena*, as well as *Entamœba coli*, ranges from 1,060 to 1,070, the cysts rise to the upper layer of water. This layer is removed by means of a sterile pipette, and the cysts are deposited in a sterile test-tube. It is necessary to have sterile apparatus. Distilled water is added until the specific gravity is 1,050. The mixture is then centrifuged. Masses of the cysts will be found in the sediment. These packed cysts are placed for 5 minutes in a 2 per cent hydrochloric acid solution in order to destroy the bacteria adhering to them, without, however, damaging the cysts. The acid is removed by centrifugation. The cysts are then introduced into the water of condensation of the medium described below. The culture is kept at a temperature of 28–30°C. The temperature has a great influence upon the reproduction of the cysts; when it falls below 28°C. reproduction cannot take place. If the temperature is maintained at body heat, the cysts die on the 2nd day. After the cysts have changed into the vegetative forms it is well to keep the temperature under 28°C. in order to inhibit bacterial growth.

[1] An electric centrifuge is not suitable, as its high speed frequently injures the cysts.

A useful culture medium consists of a mixture of two parts of 2 per cent agar and one part of defibrinated horse blood. The agar is placed in an autoclave until it is completely dissolved, after which it is cooled at a temperature of 60°C. While it is still fluid, the defibrinated horse blood is added. The mixture is then slanted and cooled. The success of the experiment depends upon external, as well as internal factors with reference to the cysts; *i.e.*, the resistance of the cysts and the state of the culture medium in regard to putrefaction.

Since November of this year, the cultivation experiment has been repeated thirty times in the manner described above. Three of eleven experiments made on *Entamœba tetragena*, and seven of nineteen on *Entamœba coli* were successful.

After a definite period of time, a small portion of the material was taken from the medium and the amebæ were examined both in their natural state on a warmed stage, and in fixed and stained preparations. Fixation was done in the usual manner in sublimate alcohol; staining was done with iron-hematoxylin, according to Heidenhain and Delafield. Examinations of the living and prepared amebæ were made at first every 5 hours, later twice a day, in the morning and the evening. In the film preparations, it is often difficult to make the material adhere to the cover-glasses, but this difficulty was obviated by placing egg albumin glycerol upon the cover-glasses.

As the morphological changes of the two amebæ resemble each other, they are described together.

Morphological Changes of Entamœba tetragena and Entamœba coli during Reproduction.

Reproduction from these cysts takes place from the 2nd to the 4th day, particularly on the 3rd day. The newly formed amebæ die frequently from the 4th to the 6th day from the effects of putrefaction of the culture medium. Naturally not all the living cysts will reproduce —in fact, the phenomenon proceeds in a relatively small number. Sometimes the cysts die without reproducing at all, while again the process is incomplete. Under favorable conditions the majority of the cysts will reproduce readily *in vitro*. On three occasions out

of nineteen experiments, it was observed that almost all the cysts of *Entamœba coli* changed on the 3rd day into the vegetative form. It is not possible to state conclusively just which cysts will reproduce, but it is probable that only the ripe cysts of both amebæ do so, as it was observed that the unripe cysts die relatively early from the effects of putrefaction of the medium or the hydrochloric acid. Whether or not the newly formed vegetative forms are cultivable remains to be proved.

Two types of reproduction were observed: (1) After a part of the cyst wall has gradually been broken, the young ameba crawls out by means of pseudopodia (Figs. 3 to 6, 8, and 42). In the space left between the cyst membrane and the cytoplasm, light-refracting oval bodies are frequently observed (Fig. 6). It is impossible to say whether these bodies are bacteria or materials extruded from the cytoplasm. The amebæ are observed to stretch out bud-like, homogeneous pseudopodia at one or more parts of the body surface (Fig. 6). It has not been noted that these plasmic extensions become separated; they are always drawn again into the protoplasmic mass. The pictures of *Entamœba coli* presented by Casagrandi and Barbagallo, and also Werner (1908), as illustrating schizogony, should not be interpreted in this manner, as they simply represent the pseudopodia of the amebæ. This mode of reproduction is observed more frequently in *Entamœba coli* than in *Entamœba tetragena*. It may be explained by the fact that the cyst wall of *Entamœba coli* is relatively thicker than that of *Entamœba tetragena*. (2) After the cyst wall has gradually disintegrated and finally disappeared, the ameba flows out by means of its pseudopodia. (*a*) Upon the dissolution of the wall there remains no space between the membrane and the cytoplasm (Figs. 32 and 72). This is the mode of reproduction followed by the majority of both these amebæ. (*b*) A space, small or large, is formed between the outer membrane and the cytoplasm (Figs. 9, 10, 43, and 60). After the disintegration of the wall the ameba emerges. The dimensions of the space thus formed differ greatly, the largest observed being about half the size of the cyst (Figs. 9, 10, and 60).

The young ameba has never been observed to move in its covering, but in fresh preparations slow rolling of the entire cyst against the slight flow in the culture fluid has been noted. It is not clear whether

this is to be ascribed to an independent movement of the cysts or to a bacterial cause.

It is noteworthy that in the case of dead cysts, plasmolysis frequently causes the cytoplasm to shrink from the wall of the cyst, creating a space such as is found in living cysts. It is possible, however, to distinguish readily the dead from the living cysts by morphological characteristics. The living cyst is pale and markedly light-refracting. Its cytoplasm is homogeneous or finely granulated, and it never takes the eosin stain. The dead cyst is more or less light-refracting, its cytoplasm is generally irregular, in large granules, and it appears rigid. The dead cysts which refract light slightly can be stained with eosin while those which are markedly light-refracting do not take the stain.

Not infrequently one encounters a *coli* cyst containing eight nuclei, the cytoplasm of which consists of inner and outer granular layers (Fig. 1). In fixed preparations, the inner layer is seen to consist of a fine network; it stains somewhat more intensely than the outer layer. Often the inner layer has at the periphery a membranous structure which sharply differentiates it from the peculiarly large meshed cytoplasm (Figs. 11 and 12). Upon removal of the cyst membrane, the inner layer, which is markedly light-refracting, escapes (Fig. 13). It is questionable whether or not this structure represents a living individual. This phenomenon, which resembles the endogenous development of *Entamœba tetragena* formerly described, was observed *in vitro* on two occasions.

In spite of numerous attempts, we did not observe the emergence from a single cyst of several daughter amebæ which corresponded to the number of nuclei of the cyst under observation. On the basis of these experiments it may be stated that only a single vegetative individual emerges from the parent cyst. The mature cyst of *Entamœba tetragena* possesses as a rule four nuclei (Fig. 41), and that of *Entamœba coli* eight (Figs. 1 and 2). For this reason, several investigators hold with Schaudinn that four or eight daughter amebæ emerge from the parent cyst, but it became clear from these experiments that the assumptions of these investigators are erroneous.

Occasionally one encounters a *coli* cyst which has its cytoplasm divided by a vacuole into a number of unequal parts (Fig. 14). The

number of these parts does not correspond to the number of nuclei contained within the cyst; some parts may harbor nuclei, while others do not. Hence it is evident that there is no process of schizogony present. Fig. 15 gives the impression, gained chiefly by the shrunken membrane of the dead cyst, that division of the cytoplasm is about to take place. On the other hand, not infrequently one finds dead cysts the granular cytoplasm of which is divided into a number of unequal parts. Probably Darling's illustration of schizogony is based on this phenomenon. Fig. 63 shows a number of ameba-like organisms cultivated *in vitro*. In the body of the cell lies an oval, readily stained portion. These organisms, which like the malaria plasmodia later divide by schizogony into a number of daughter individuals, resemble the so called schizogony forms of *Entamœba coli*, as described by Schaudinn. But the number of daughter amebæ issuing from the mother is always restricted to eight.

The vegetative forms of both amebæ as observed *in vitro* were never smaller than eight microns. With regard to the small amebæ found in feces which have been reported as *Entamœba coli*, we agree with Hartmann. It is questionable whether these forms are *Entamœba coli*. Werner gives the diameter of the smallest forms of *Entamœba coli* as five microns. Darling gives the size of *Entamœba tetragena* as six microns. But as neither Werner nor Craig presents any illustrations of these small forms, we cannot affirm them to be *Entamœba coli*. Casagrandi and Barbagallo give ten microns as the size. Small amebæ of this kind which have been observed frequently are shown in Figs. 35, 61, and 62. It is, however, our opinion that these forms have not arisen through schizogony, but rather by the mode of reproduction described above. We observed previously that the flagellate forms of *Trichomonas* and *Cercomonas* change into the ameboid form in a mixture of serum and Ringer's solution. The ameboid form is always smaller than the smallest form of *Entamœba coli*. In movement and structure, in fact in everything except size and nuclear structure, it shows great resemblance to the vegetative form of the *coli* ameba. Hence it may be assumed that the *coli* ameba is frequently confused with these flagellates.

The nucleus of the living *coli* cyst, because of its chromatin mass, is more readily visible than that of the *tetragena* cyst. In fresh prepa-

rations, the cytoplasm becomes markedly light-refracting and opaque shortly before or after reproduction, which, as in the case of *Amœba albida* of Nägler, renders morphological examination difficult (Figs. 6, 7, and 8). In the cytoplasm granules and vacuoles are also frequently present, so that it is not possible to establish the number of nuclei. But a number of nuclei can be plainly observed in the developed amebæ (Figs. 39 and 40); in most *Entamœba coli* they are closely massed (Fig. 38). In the cytoplasm of the newly formed ameba there are at first few granules, and it appears to be without structure. The differentiation of ectoplasm and endoplasm is indistinct, but later this becomes plain. Rod-like or oval granules, as well as numerous bacteria, are frequently seen in the cytoplasm. Whether these granules represent chromidia or bacteria has not been determined.

The movement of the newly formed amebæ is as a rule not vigorous and proceeds by means of sac-like pseudopodia. No definite direction of movement is seen. It happens not infrequently that in reproduction and under suddenly changed conditions—such as a change of temperature or culture medium—the individual puts out finger-tip pseudopodia (Figs. 6, 52, 53, 55, and 66). As the *coli* ameba is less markedly light-refracting than that of *tetragena*, it is often overlooked. The ectoplasm of *Entamœba coli* has frequently less light-refracting properties than the endoplasm, while these conditions are reversed with respect to *Entamœba tetragena*.

The morphological changes of both amebæ which have been followed in a series of fixed preparations are described below. The nuclei of the cysts extrude large masses of chromatin into the cytoplasm. The chromatin is not divided into chromidial bodies, but lies in fine granules within the cytoplasm. In the beginning the cytoplasm consists of a fine network, later of a coarse, irregular network which stains intensely with hematoxylin (Figs. 21, 25, and 53). At times the preparation takes the hematoxylin and orange stain intensely and is covered with precipitate. The meshes themselves are homogeneous and do not stain. *Entamœba tetragena*, on the whole, does not stain so well as *Entamœba coli*, and its reticular structure is more delicate. The ability to absorb the stain appears to be an individual characteristic; occasionally one sees amebæ which stain quite as well as the small

vegetative forms found in feces (Figs. 17, 45 to 47, 49, 54, and 55), and there are amebæ which stain very intensely with hematoxylin, so that the nucleus is hardly recognizable. In such structures it was possible in two stained preparations to observe the homogeneous pseudopodium (Fig. 36). Sometimes one sees intensely stained amebæ of a dirty color with joint, compact nuclei in the environment of which there is a light zone (Fig. 27). When these amebæ are decolorized, the nuclei are plainly visible. Within the nuclei there is an increasing accumulation of chromatin. The peripheral chromatin zone of the nucleus gradually broadens in the course of the cyclic process, and large masses of chromatin are then extruded into the cytoplasm. The outer chromatin is particularly well developed at this stage, and it is arranged in a half moon or ring-shaped form. The nuclear network is not very distinct. In advanced stages the nuclei are often pycnotically stained because of the marked chromatin accumulation, so that the inner structure is no longer distinguishable.

The multiple nuclei of the cyst then approach one another gradually and merge (autogamy), forming one or two fusion nuclei (syncaryons). The number of nuclei participating in the fusion varies, but as a rule there is a merging of three or four, rarely six nuclei. Four is the usual number in *Entamœba coli*, and from two to four in *Entamœba tetragena*. We observed several *coli* cysts in which two or three nuclei were gathered close together at two points in the cytoplasm (Figs. 32, 33, and 72), and again newly emerged vegetative forms with large nuclei (Figs. 34 and 73). These pictures show that syngamic nuclear union may take place simultaneously at two points within the same individual. This process has never been observed in *Entamœba tetragena*.

As already stated, it has been possible to establish the fact that autogamy takes place in both *Entamœba tetragena* and *Entamœba coli*, not only between two nuclei, but also it may involve more than that number in order to produce a syncaryon, while in the case of other protozoa this process is operative only between two nuclei. In order to distinguish these types of autogamy, the terms simple and polynuclear have been devised. The latter may be further classified as trinuclear, tetranuclear, and pentanuclear autogamy.

Morphological investigation of the structure of the nucleus in reproduction is as a rule very difficult, because of the abundance of

chromatin. But in nuclei relatively poor in chromatin one is able to observe the merging of the nuclear membranes and the formation of a common membrane about the nuclei. The peripheral chromatin masses are then seen to fuse. For this reason at this stage there is seen a syncaryon which still retains the form of the daughter nuclei, so that it is possible to determine the number of daughter nuclei participating in the process of autogamy. Figs. 18, 20, and 68 show four nuclei fusing to form a syncaryon. Figs. 21 to 25, 52 to 56, 66, and 76 illustrate trinuclear autogamy. Figs. 44 to 48 and 75 show the autogamy of two nuclei with sparse chromatin material. It is difficult to follow the changes in the caryosome of the daughter nuclei in reproduction, because of the small amount of chromatin contained in the caryosome, which appears indistinct. It seems probable, however, that the fusion of caryosomes occasionally takes place within the nucleus, for not infrequently one observes what appears to be a fusion of two caryosomes (Figs. 4 and 48). Diminution of the chromatin follows complete union of two or more nuclear parts. The large mass of chromatin of the syncaryon is then dissolved gradually, leaving a residue of peripheral chromatin and the newly formed caryosome. A meshy structure is formed between the outer chromatin and the caryosome.

The syncaryon is at first irregularly formed, and appears flattened, polygonal, almond-shaped, or in the form of a cross, giving the impression of fused nuclei. There is no distinct outline to the nucleus in this stage. With the lessening of the nuclear chromatin, the syncaryon assumes a spherical shape (Figs. 26 and 57), and later it has a structure resembling that of the vegetative forms found in feces (Figs. 31, 58, and 62). The nuclei not participating in the process of autogamy were found to degenerate before or after the syngamic nuclear union; they finally disappear (Figs. 4, 16, 22, 43, 46, and 55) or are extruded from the organism as foreign bodies (Fig. 51).

Similar processes are observed in other protozoa. Von Prowazek has described a sexual cycle in the cyst of *Bodo lacertæ* in which two of the total number of six gametes are autogamically united, while the other four degenerate and disappear. Glaeser noted in regard to the tetranuclear cyst of *Amœba mira* that three nuclei perish, and that the remaining one evidently represents the gamete. Nägler in the

case of the cyst of *Amœba albida* observed a similar process, which consisted in the union of two gametes and the disappearance of the other two nuclei.

The best known case of autogamy is the conjugation of *Actinosphærium eichhorni* so well described by Hertwig. In this polynuclear heliozoan, reproduction is preceded by destruction of a part of the cell nucleus. Within the mother cyst the number of nuclei, from 20 to 500, is reduced to about 5 per cent of the total number. The disappearance of the nuclei is effected by processes of dissolution and resorption. During the past year we have not found any vacuoles in the tetranuclear cysts of *Trichomonas intestinalis* and we were able to determine in the cultivation of these cysts the development of a young ameboid form, the fusion of a number of nuclei, and the degeneration of others, as in *Entamœba tetragena*.

The ejection of the nuclei is a phenomenon which had already been observed by a number of investigators. Wenyon established the extrusion of superfluous nuclear substances with regard to *Entamœba muris*. Von Prowazek demonstrated a number of times the expulsion of nuclei in the vegetative form of *Entamœba williamsi*, and gave an illustration. He described this stage as a particular form of rejuvenation, asexual in character, which is not rare among protozoa. In this procedure, in our opinion, there is solely an expulsion of useless substances. Fig. 16 shows a *coli* cyst which has two degenerated and four closely joined nuclei. Fig. 30 illustrates a vegetative form possessing a large syncaryon and two residual nuclei. It is not difficult to interpret this picture: a number of the closely joined nuclei unite, while the other nuclei, which do not participate, shrink and degenerate. This picture is sometimes encountered in material obtained from the feces of cats infected with the cysts.

These processes of autogamy take place at the reproductive stage or shortly after; *i.e.*, in the newly emerged vegetative forms, as in the case of *Amœba mira*, according to Glaeser. In a number of vegetative forms the nuclei do not conjugate completely. Sometimes the syngamic nuclear union is already present in the cyst, and again one finds cysts possessing developed syncaryons. On the whole it may be said that the nuclei approach one another in the cystic stage, and they unite after reproduction takes place. Hence shortly after reproduc-

tion, the nuclei present the picture of the middle stage of autogamy. Fig. 5 shows a *coli* ameba, which still has six nuclei, emerging from its burst shell. Fig. 43 illustrates an *Entamœba tetragena*, of which two nuclei remain quite isolated, although a part of the membrane has already loosened. Fig. 60 shows a *tetragena* cyst with two nuclei in process of conjugation. Fig. 42 illustrates the emerging of the *tetragena* ameba from the capsule after the completion of the syngamic nuclear union. Fig. 57 shows a newly created *tetragena* ameba, produced by autogamy. Fig. 50 illustrates a newly created vegetative form with four nuclei lying close together, without, however, fertilization. As the syngamic nuclear union is a slow process, it is possible to obtain in stained preparations numerous pictures illustrating autogamy.

The newly created ameba frequently attains the size of the cyst, but again it may be only half as large. Hence, it is obvious that the vegetative form of the *coli* cyst shortly after reproduction is larger than that of *Entamœba tetragena*, inasmuch as the *coli* cyst as a rule is larger than that of *tetragena*. These small amebæ shortly after reproduction take in various bacteria for food (Figs. 22, 44, and 49); and on the 4th day of development they attain the size of the normal vegetative forms found in feces. As during this stage bacterial proliferation is marked, the culture medium undergoes decomposition and becomes chocolate-colored. The amebæ then develop numerous vacuoles, and their nuclei shrink and degenerate (Fig. 59), until they finally burst. On the 5th day of the experiment one rarely encounters large amebæ and the small forms have wholly disappeared.

On the basis of the results described above the conclusion was drawn that the daughter nuclei in the cyst stage correspond exactly to the gamete nuclei of other protozoa. The difference between these gametes and those of other protozoa consists only in the fact that these gametes show no differentiation between male and female, and are enclosed in a common cytoplasm. We have here an example of primitive sexual fertilization. Schaudinn and other investigators have established the fact that the change into a chromidial cell is a normal process in the formation of gametes in a number of protozoa. This may also be assumed with regard to *Entamœba coli* and *Entamœba tetragena*. The fact that the nucleus of the vegetative forms extrudes large masses of chromatin during the formation of the cyst,

and that this chromatin is then developed into small daughter nuclei (gametes), agrees entirely with the views of Schaudinn. We have previously described how in the cyst formation of the atypical forms of *Entamœba tetragena*, the chromatin, or chromidia, extruded from the nucleus into the cytoplasm, later form from one to four daughter nuclei, as has been proved by Popoff to be the case in *Amœba minuta*. The process is in accord with the phenomenon observed in Rhizopoda in which the nuclei of the gametes are reconstructed from chromidia. These facts afford proof that the daughter nuclei of the cysts correspond to the gamete nuclei. Schaudinn has assumed that all protozoan cells are binuclear. He says:

> While in the case of the Infusoria, in Polystomella, Centrophyxis, and Chlamydophyrs, the sex substance of the nucleus during the vegetative period develops separately from the metabolism nucleus, and arises out of a special nucleus or chromidial mass, in the case of *Entamœba coli* it is united with the vegetative nuclear substance in one nucleus. A separation takes place only just before conjugation, when the substance of the metabolism nucleus, or the nucleus itself, is extruded or otherwise destroyed. A chromidium arises and exists for a short period. Out of this the sex nuclei are differentiated.

Nägler also observed in the case of the cyst of *Amœba albida* that a separation of the generative and vegetative nuclei precedes autogamy. According to our observations, chromidial formation in the case of *Entamœba coli* and *Entamœba tetragena* is found more frequently in the gamete stage than in fertilization, and with the exception of a few cases it is not possible to demonstrate unequivocally a reductional division before fertilization takes place. One observes, however, that the nuclei which do not participate in autogamy become disintegrated and that the nuclear chromatin passes into the cytoplasm. In the case of *Entamœba tetragena*, which is related to *Entamœba coli*, the chromidia and the chromatin play a large part in the formation of the gametes, and also on the other hand in the vegetative function. On the basis of these observations we are unable to confirm Schaudinn's theory of nuclear dualism with respect to the protozoan cell. It appears to be logical to assume that there are two kinds of chromatin, as already asserted by Doflein; *i.e.*, idiochromatin and somatochromatin.

On two occasions the author encountered in the culture medium a *coli* cyst having more than eight nuclei (Fig. 37). These nuclei are arranged in pairs, and the pictures show that following the disappearance of a number of nuclei, those remaining rearrange themselves in groups of two. It has not been determined whether or not this represents a reduction process. Hartmann and other investigators have recorded *coli* cysts with more than eight nuclei.

Schaudinn has described a complex nuclear change which occurs in the formation of the cyst of *Entamœba coli*. He claims that syngamic nuclear union occurs only in the tetranuclear stage and is the result of conjugation and the metagamic reproductive processes of a cyst with eight nuclei. In support of Schaudinn's view of autogamy, we have the claims of Wenyon with respect to *Entamœba muris*, which is now assumed to be identical with *Entamœba coli*. Hartmann and Withmore on the basis of their own experience did not admit the claims of Schaudinn and Wenyon, but came to the conclusion that no autogamic process of fertilization within the cyst had been observed. We concur in this view of Hartmann. Wenyon claimed to have made consecutive observations upon living cysts and gave as proof a number of consecutive illustrations of the same ameba. But he described autogamy very briefly, as did Schaudinn, and presented no illustrations of importance. For instance, he did not illustrate the conjugation of the four pairs of cells. Concerning autogamy he wrote:

"The two nuclei of each pair then apparently fused, producing again a cyst with two nuclei. These two nuclei then began to increase in size and almost immediately divided to form four nuclei."

It is clear that no unequivocal proof exists that autogamy has been observed by Wenyon in a living individual. Again he wrote:

"The duration of the spindle formation and conjugation was at most only ten minutes, and this explains the difficulty of finding these stages in fixed preparations."

In the case of other amebæ no such rapid phenomenon of isogamy, anisogamy, or autogamy is known at the present time. He appears to describe an unusual case.

Nägler states that it is impossible to follow the syngamic nuclear union in living specimens of *Amœba albida*, as the process requires

at least 24 hours for its completion. This is also true of *Entamœba coli*. In the cases observed by us, the phenomenon of autogamy required quite a long period of time, and it was possible to follow easily the various stages in the fixed preparations. If the theories of Schaudinn and Wenyon were correct, there would be two formations of gametes in one cyst, which is inadmissible. For that reason their views seem unsound.

A similar statement may be made concerning *Entamœba tetragena*. We believed, like Schaudinn, that, preceding the formation of four daughter nuclei in the cyst, processes similar to those of *Entamœba coli* take place with respect to *Entamœba tetragena*, because of the relation existing between the two amebæ; but we were unable to establish these processes in the encystment of dysentery amebæ *in vitro*. Hartmann claimed at an earlier date to have discovered the process of autogamy in the *tetragena* cyst, but a subsequent study of the subject convinced him that he was not concerned with examples of autogamy, but with a degenerated form. Since that time no one has reported the process with regard to *Entamœba tetragena*. It must be stated, however, that the phenomenon of autogamy in *Entamœba coli* as well as *Entamœba tetragena* is not seen in the early cystic stage, but during reproduction.

To assume the phenomenon of schizogony for *Entamœba coli* also seems erroneous. As already stated, the illustrations of Casagrandi and Barbagallo, as well as those of Werner, do not illustrate schizogony, but the emergence of a *coli* ameba from its cystic membrane. The schematic illustration of Wenyon is based upon conjecture. The illustration of a *tetragena* cyst given by Darling was probably that of a dead cyst, in parts of which the granular cytoplasm had been accidentally separated.

The possibility of heterogamy in the development of these two amebæ will be considered briefly. Previously we were able to demonstrate *in vitro*, in the case of the large chromidial forms of *Entamœba tetragena*, the fusion of two amebæ, as Hartmann and Werner claim to have observed in the case of *Entamœba histolytica*. But this fusion has been encountered very rarely, and a union of the nuclei has not been observed. Hence it is not proper in that case to speak of conjugation, but one should rather regard it as plasmogony. More-

over, we have frequently observed three specimens of the small vegetative forms of the dysenteric amebæ hanging together by thin protoplasmic stems. Such pictures do not represent conjugation, but rapid cell division. Therefore, it was not possible for us to demonstrate heterogamy *in vitro* in the period of time required by the large vegetative form for its changes into the cyst. If heterogamy were to take place in amebæ, we would have to look for it during the period in which the cyst changes into the large vegetative form. Assuming, according to Schaudinn, that a number of daughter amebæ emerge from a single cyst by schizogony, one must furthermore consider that they conjugate in pairs to form a copula, as described by Mercier with regard to *Entamœba blattæ* and by Popoff for *Amœba minuta*. But up to the present time we have not observed this form of schizogony, *in vitro*, or such pictures of conjugation. Von Prowazek with respect to *Entamœba williamsi*, which is related to *Entamœba coli*, indicated heterogamy. He stated:

> A number of times we observed very small amebæ lying close together. In the preparations binuclear stages were seen, in which the nuclei were distinct, and as there were also indications of a reduction process, the assumption of heterogamy seems warranted for this entameba.

These observations of von Prowazek are open to question, and his illustrations which might be interpreted as showing a process of fertilization and division by reduction, correspond to Figs. 30 and 35. They do not illustrate heterogamy, but rather autogamy. The origin of these small nuclei is to be attributed to the fact that the daughter nuclei which might participate in the autogamy finally shrink. Similar pictures are found frequently in the nuclear division of the atypical vegetative forms of *Entamœba tetragena*, shortly before encystment.

Development in Vitro of Entamœba tetragena.

In 1918 (*a*), the author reported in his first communication on the subject that the large vegetative form of dysenteric ameba always passes into a small vegetative form, and produces at times a tetranuclear cyst *in vitro*, and that more or less proliferation takes place when feces showing blood-tinged mucus are kept *in vitro*. In this second communication the writer shows that it is possible, by follow-

ing a definite method, to grow *in vitro* the large vegetative forms from the cysts. The two papers, in which different culture media are described, together present the entire developmental cycle of *Entamœba tetragena in vitro*.

CONCLUSIONS.

1. The vegetative forms of amebæ may be developed *in vitro* from the cysts.

2. The cysts of *Entamœba tetragena* and *Entamœba coli* each produce only a single vegetative individual. It has not been observed *in vitro* that a number of daughter amebæ, corresponding to the number of nuclei present in the cyst, emerge from the mother cyst, as claimed by Casagrandi and Barbagallo, Schaudinn, and a number of other investigators.

3. The daughter nuclei in the cystic stage correspond to the gamete nuclei of other protozoa. The syngamic nuclear union in the case of these two amebæ does not take place in the tetranuclear stage of the cyst, as claimed by Schaudinn and Wenyon, but during reproduction.

4. The process of autogamy of both amebæ is not restricted to two nuclei; it may involve more than two, which unite to form a syncaryon. In the case of all other protozoa, autogamy is restricted to two nuclei.

5. In order to distinguish the two types of autogamy, one has been termed simple autogamy and the other polynuclear autogamy. The latter may again be classified according to the number of nuclei involved, as trinuclear and tetranuclear autogamy.

6. The majority of cysts treated for 5 minutes in a 2 per cent solution of hydrochloric acid survives.

7. It was possible to demonstrate *in vitro* the developmental cycle of *Entamœba tetragena*.

8. No evidence of heterogamy has been observed *in vitro* in *Entamœba tetragena* or *Entamœba coli*.

9. The vegetative form of *Entamœba tetragena*, at a certain stage of its life cycle *in vitro*, shortly after its formation, has not only one or two, but may have as many as three or four nuclei.

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EXPLANATION OF PLATES.

PLATE 29.

FIGS. 1 to 20. *Entamœba coli.*

FIG. 1. Cyst with eight nuclei. The cytoplasm consists of outer and inner granular layers.

FIG. 2. Ripe cyst containing eight nuclei.

FIGS. 3 to 5. Reproduction of cysts. (Stained preparations.)

FIGS. 6 to 8. Reproduction of cysts. (Fresh preparations.)

FIGS. 9 and 10. Cysts having a small zone between the cyst membrane and the cytoplasm. (Stained preparations.)

FIG. 11. The inner layer of the cytoplasm shows at the periphery a membranous structure, which serves to differentiate the coarse meshed cytoplasm from the inner layer. (Fresh preparation.)

FIG. 12. The same. (Stained preparation.)

FIG. 13. Rupture of cyst membrane shown in Figs. 11 and 12.

FIG. 14. Cyst showing eight nuclei. The cytoplasm is divided by a vacuole into a number of unequal parts.

FIG. 15. Dead cyst. The wall of the cyst shows folds caused by shrinkage. (Fresh preparation.)

FIG. 16. Cyst shortly before reproduction. Four nuclei are intact; three nuclei are degenerated.

FIGS. 17 to 20. Tetranuclear autogamy. Fig. 20 is a vegetative form with syncaryon.

PLATE 30.

FIGS. 21 to 40. *Entamœba coli.*

FIGS. 21 to 25. Vegetative forms, showing trinuclear autogamy.

FIG. 26. Vegetative form with syncaryon.

FIGS. 27 to 29. Vegetative forms showing autogamy involving more than four nuclei.

FIG. 30. Vegetative form with syncaryon and two degenerated gamete nuclei.

FIG. 31. Newly created vegetative form.

FIGS. 32 and 33. Vegetative forms showing autogamy at two points. Fig. 32 is a vegetative form with residual cyst membrane.

FIGS. 34 and 35. Vegetative forms with two syncaryons.

FIG. 36. Vegetative form deeply stained with hematoxylin.

FIG. 37. Cyst having more than eight nuclei.

FIGS. 38 to 40. Reproduced vegetative forms. (Fresh preparations.)

PLATE 31.

FIGS. 41 to 62. *Entamœba tetragena.*

FIG. 41. Tetranuclear cyst of *Entamœba tetragena.*

FIG. 42. Reproduction of *tetragena* cyst, upper part showing cyst membrane, lower portion the vegetative form.

FIG. 43. Cyst shortly before reproduction. Two nuclei are intact, while two others are degenerated.

FIGS. 44 to 49. Binuclear autogamy. Fig. 44 is a vegetative form with three vacuoles, containing bacteria. Fig. 49 is a vegetative form with syncaryon produced by binuclear autogamy.

FIG. 50. Vegetative form with four nuclei.

FIG. 51. Vegetative form, showing extrusion of a gamete nucleus.

FIGS. 52 to 56. Vegetative forms, showing trinuclear autogamy. Fig. 56 is a vegetative form which has completed trinuclear autogamy.

FIGS. 57 and 58. Vegetative forms with syncaryons.

FIG. 59. Degenerated form observed *in vitro* on 4th day of experiment.

FIG. 60. Cyst in autogamy.

FIGS. 61 and 62. Small vegetative forms.

FIG. 63, *a*, *b*, and *c*. Ameboid organisms.

PLATE 32.

FIGS. 64 to 71. *Entamœba coli.*

FIGS. 64 and 65. Reproduction of *Entamœba coli.* × 900.

FIG. 66. Vegetative form, showing trinuclear autogamy. × 900.

FIG. 67. Vegetative form, showing tetranuclear autogamy. × 900.

FIG. 68. Vegetative form with syncaryon produced by tetranuclear autogamy. × 900.

FIG. 69. Vegetative form, showing autogamy of more than four nuclei. × 900.

FIG. 70. Same as Fig. 30. × 900.

FIG. 71. Vegetative form with a syncaryon. × 900.

PLATE 33.

FIGS. 72 and 73. *Entamœba coli.* × 900.

FIG. 72. Same as Fig. 32.

FIG. 73. Vegetative form with two syncaryons.

FIGS. 74 to 77. *Entamœba tetragena.* × 900.

FIG. 74. Reproduction of cyst, upper portion showing membrane, lower portion a reproduced individual. × 900.

FIG. 75. Vegetative form, showing binuclear autogamy. × 900.

FIG. 76. Vegetative form, showing trinuclear autogamy.

FIG. 77. Two new vegetative forms.

PLATE 34.

Development of *Entamœba tetragena in vitro.*

1 to 17. Typical forms.

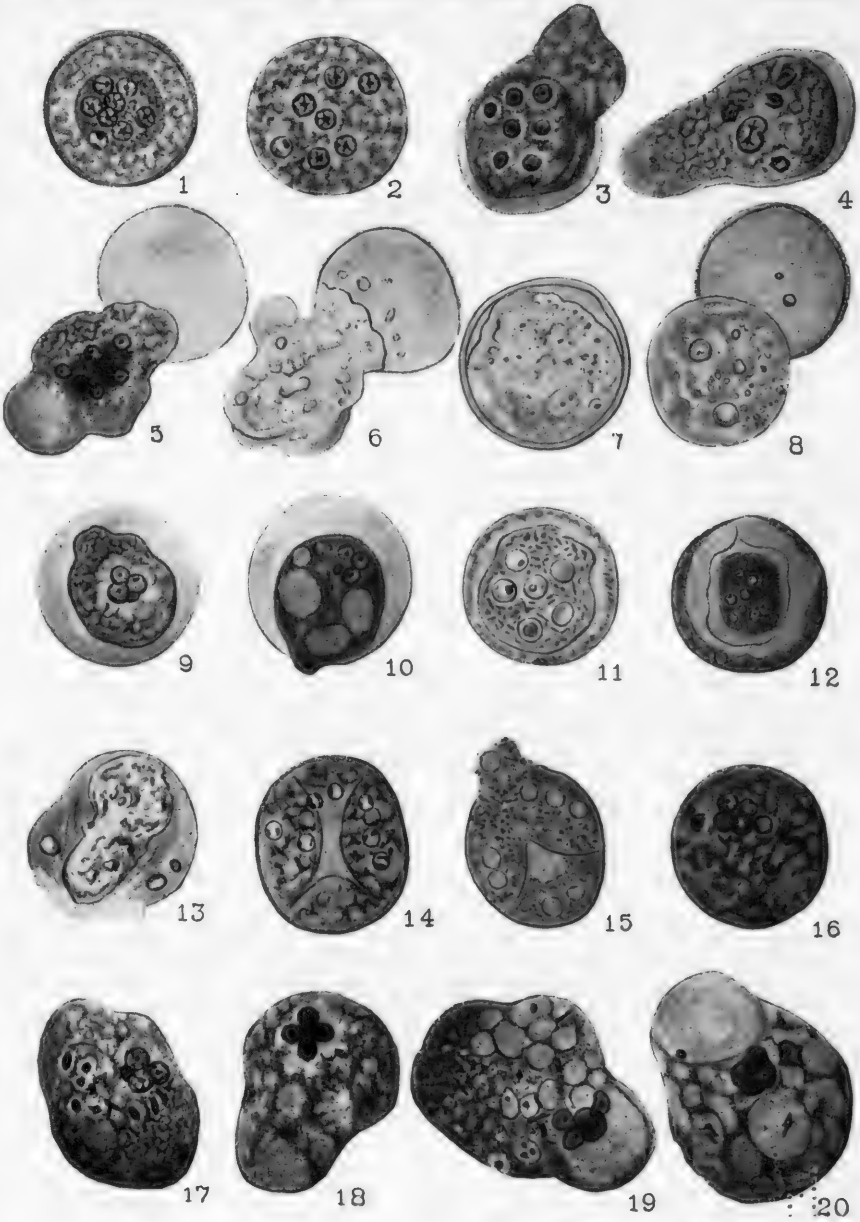
18 to 27. Atypical forms.

1. Large vegetative form.
2. Transitional form.
3. Small vegetative form.
4. Small vegetative form (chromidial form).

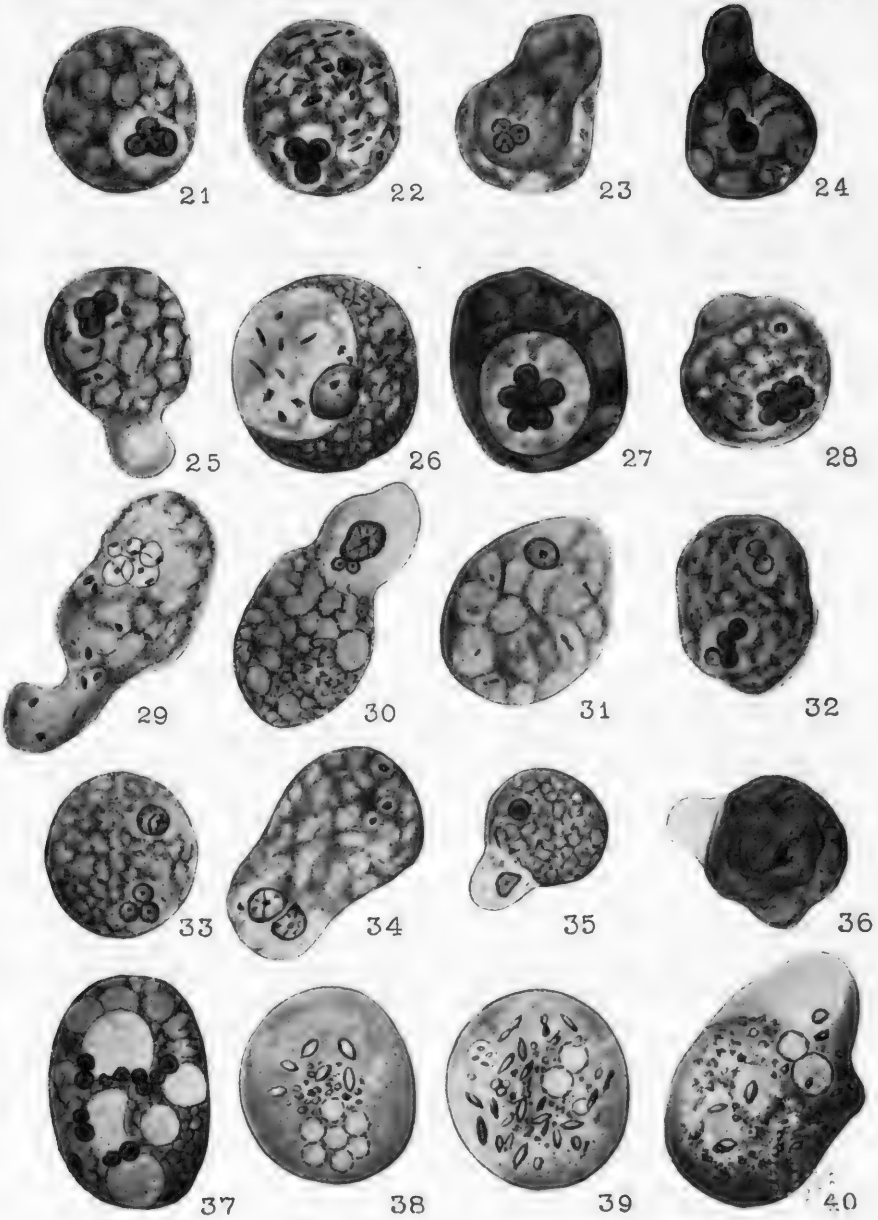
5 to 10. Cysts.

5. Mononuclear cyst with central vacuole.
6. Mononuclear cyst. Mitosis.
7. Binuclear cyst.

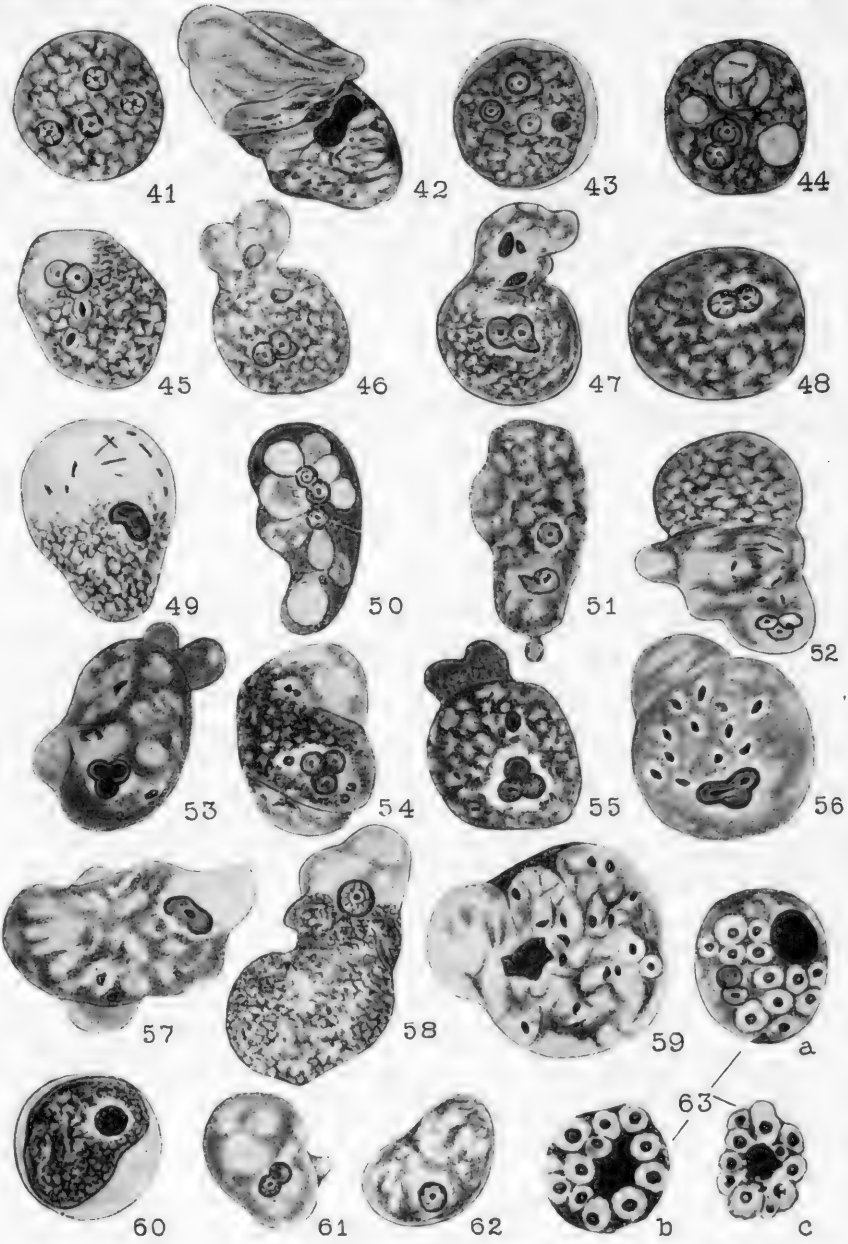
8. Trinuclear cyst. Mitosis.
9. Tetranuclear cyst.
10. Tetranuclear cyst. Two nuclei are intact, while two others are degenerating.
11. Reproduction of cyst. Binuclear autogamy.
12. Reproduced vegetative form. Binuclear autogamy.
13 to 15. Trinuclear autogamy.
15. Reproduced vegetative form with syncaryon and one shrunken nucleus.
16 and 17. Young vegetative forms.
18 to 21. Cyst formation of atypical forms. Multiple nuclear division within the nuclear substance.
22 to 25. Cyst formation of atypical forms. Multiple nuclear division outside the degenerating nucleus.
26 and 27. Degenerated atypical forms.
28 and 29. Reproduction: 28, by division; 29, by a form of budding.



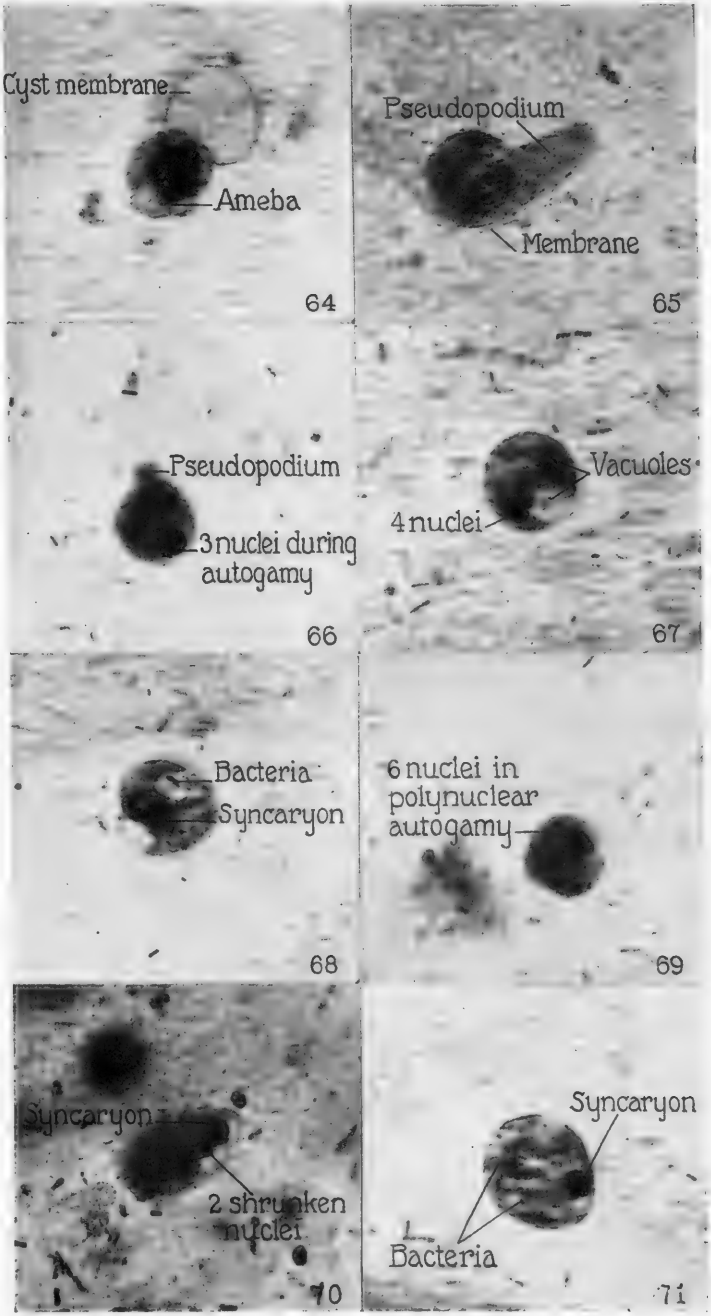
(Yoshida: *Entamœba tetragena* and *Entamœba coli*.)



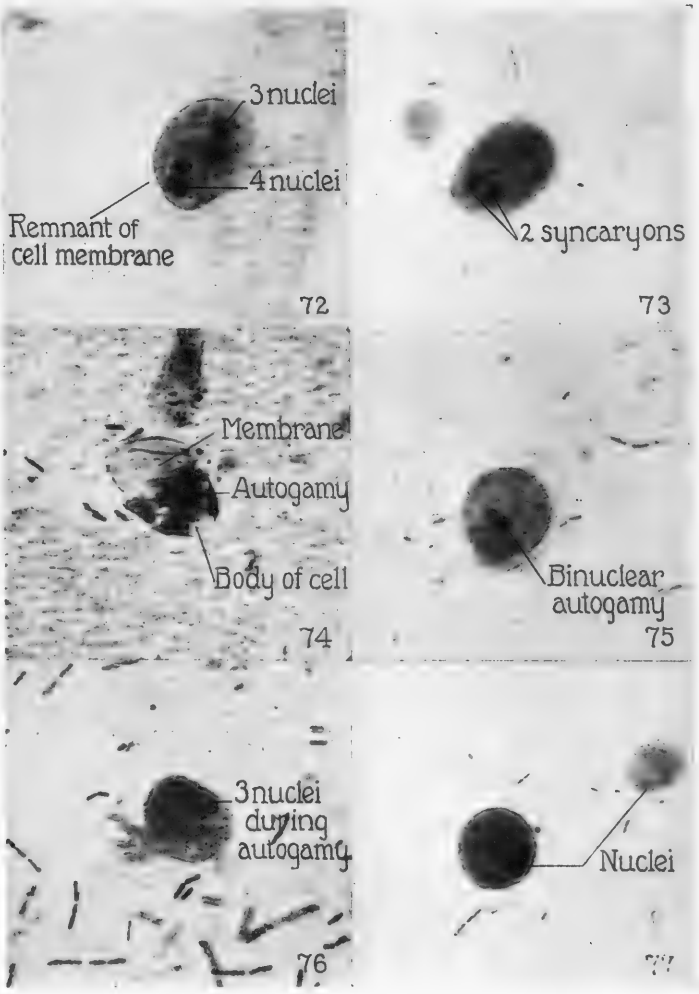
(Yoshida: *Entamœba tetragena* and *Entamœba coli*.)



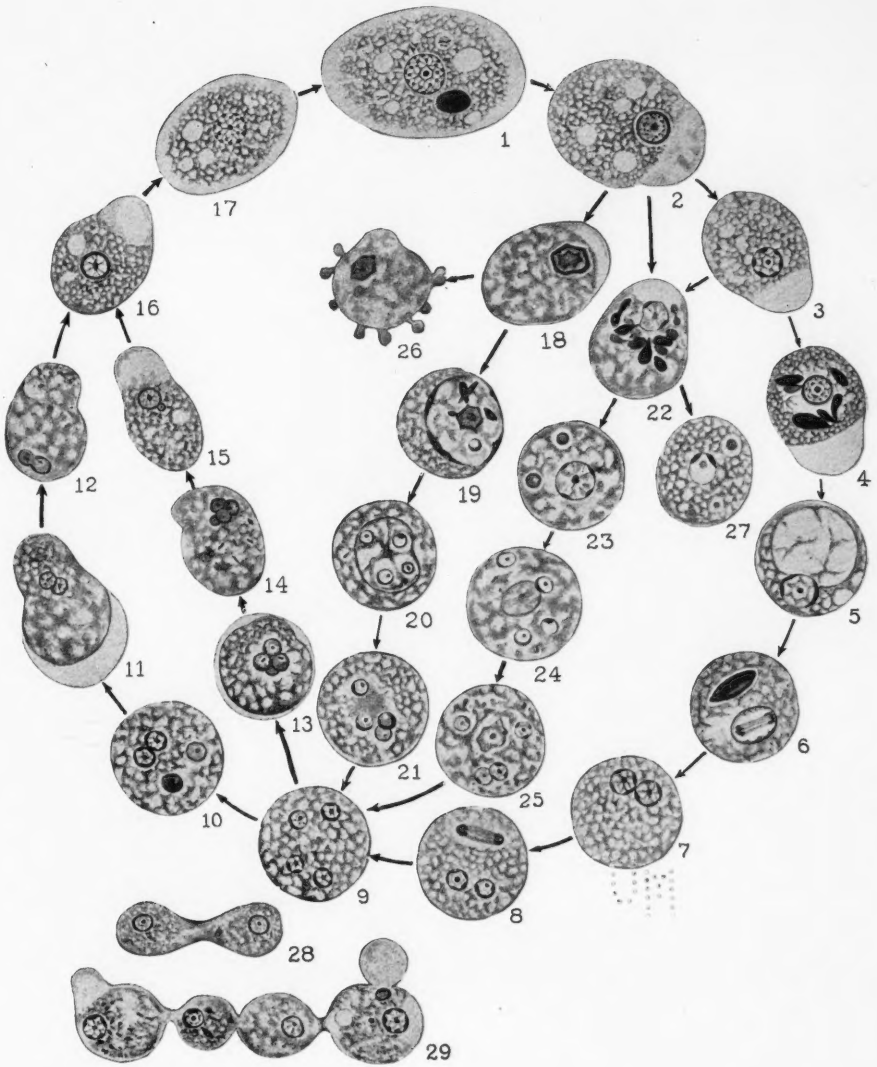
(Yoshida: *Entamœba tetragena* and *Entamœba coli*.)



(Yoshida: *Entamœba tetragena* and *Entamœba coli.*)



(Yoshida: *Entamœba tetragena* and *Entamœba coli*.)



(Yoshida: *Entamœba tetragena* and *Entamœba coli*.)